ANTISENSE MODULATION OF TRANSFORMING GROWTH FACTOR-ß EXPRESSION

INTRODUCTION

This application is a continuation of U.S. Application Serial No. 09/948,002 filed September 5, 2001, which is a continuation-in-part of U.S. Application Serial No. 09/661,753, filed September 14, 2000 issued as U.S. Patent 6,436,909, which claims the benefit of U.S. Provisional Application No. 60/154,546 filed September 17, 1999.

FIELD OF THE INVENTION

The present invention provides compositions and methods for modulating the expression of transforming growth factor-ß (TGF-ß). In particular, this invention relates to antisense compounds, particularly oligonucleotides, specifically hybridizable with nucleic acids encoding human TGF-ß. Such oligonucleotides have been shown to modulate the expression of TGF-ß.

BACKGROUND OF THE INVENTION

Transforming growth factor-ß (TGF-ß) is a cytokine which regulates biological processes such as cell proliferation, differentiation and immune reaction. It has been found to have many actions in tissue repair, stimulating the deposition of extracellular matrix in multiple ways. TGF-ß stimulates the synthesis of matrix proteins including fibronectin, collagens and proteoglycans. It also blocks the degradation of matrix by inhibiting protease secretion and by inducing the expression of protease inhibitors. It also facilitates cell-matrix adhesion and matrix deposition via modulation of expression of integrin matrix receptors, and lastly TGF-ß also upregulates its own expression. TGF-ß exists in three isoforms in mammals: TGF-ß1, -2 and -3. These function similarly *in vitro*.

Fibrosis is a pathological process, usually resulting from injury, which can occur in any organ. Excessive amounts of extracellular matrix accumulate within a tissue, forming scar tissue which causes dysfunction and, potentially, organ failure. Fibrosis can be either chronic or acute. Chronic fibrosis includes fibrosis of the major organs, most commonly liver, kidney and/or heart, and normally has a genetic or idiopathic origin. Progressive fibrosis of the kidney is the main cause of chronic renal disease. In diabetics, fibrosis within glomeruli (glomerulosclerosis) and between tubules (tubulointerstitial fibrosis) causes the progressive loss of renal function that leads to end-stage renal disease. Fibrotic lung disorders include some 180 different conditions and result in severe impairment of lung function.

Acute fibrosis is associated with injury, often as a result of surgery. Surgical adhesion represents the largest class of acute fibrosis. Surgery often results in excessive scarring and fibrous adhesions. It is estimated that over 90% of post-surgical patients are affected by adhesions. Abdominal adhesions can lead to small bowel obstruction and female infertility. Fibrosis after neck and back surgery (laminectomy, discectomy) can cause significant pain. Fibrosis after eye surgery can impair vision. Pericardial adhesions after coronary bypass surgery, fibrosis after organ transplant rejection and general scarring after plastic surgery are other examples. This represents a major unmet medical need.

Antisense and other inhibitors of TGF-ß have been used to elucidate the role of TGF-ßs in cancer, anaphylaxis, fibrosis and other conditions. As examples:

Dzau (WO 94/26888) discloses use of antisense sequences which inhibit the expression of cyclins and growth factors including TGF-$ß_1$, TGF, bFGF, PDGF for inhibiting vascular cellular activity of cells associated with vascular lesion formation in mammals. Shen et al. discloses use of

phosphorothioate antisense oligonucleotides targeted to TGF-ß2 to reduce TGF-ß2 expression in U937 cells (Bioorg. Med. Chem. Lett., 1999, 9, 13-18).

Schuftan et al. (**1999**, *Eur. J. Clin Invest., 29, 519-528*) disclose use of a2-macroglobin or antisense to TGF-ß1 to reduce extracellular matrix synthesis in cultured rat hepatic stellate cells.

Kim et al. have used antisense oligonucleotides targeted to TGF-ß1 to inhibit passive cutaneous anaphylaxis and histamine release. **1999**, *J. Immunol. 162,* 4960-4965.

Kim et al. have also used an antisense TGF-ß1 oligodeoxynucleotide to inhibit wound-induced expression of TGF-ß1 mRNA in mouse skin. Pharmacol. Res., **1998**, 37, 289-293.

Liu et al. used TGF-ß antibody or antisense to TGF-ß1 to inhibit secretion of plasminogen activator inhibitor-1 in EGR-1 regulated cells. **1999**, *J. Biol. Chem. 274,* 4400-4411.

Arteaga et al. used antibodies or antisense oligonucleotides targeted to TGF-ß2 to enhance sensitivity of cancer cells to NK cells in the presence of tamoxifen. **1999**, *J. Nat. Cancer Inst. 91,* 46-53.

Tzai et al. , 1998, *Anticancer Res., 18,* 1585-1589, used antisense oligonucleotides specific for TGF-ß1 to inhibit *in vitro* and *in vivo* growth of murine bladder cancer cells.

The role of TGF-ß in diabetic nephropathy is reviewed in Hoffman, et al.,**1998**, Electrolyte Metab., 24, 190-196. Neutralizing anti-TGF-ß antibodies or antisense oligonucleotides directed to TGF-ß1 are reported to prevent the hypertrophic effects of high glucose and the stimulation of matrix synthesis in renal cells.

Antisense phosphorothioate oligodeoxynucleotides targeted to TGF-ß3 were used by Nakajima et al. (**1998**, Japan. Dev. Biol, 194, 99-113; abstract only) and others to block

transformation of atrioventricular canal endothelial cells into invasive mesenchyme.

Chung et al. (US Patent 5,683,988) disclose and claim particular antisense oligodeoxynucleotides targeted to TGF-ß and use of these to inhibit scarring.

SUMMARY OF THE INVENTION

The present invention is directed to antisense compounds, particularly oligonucleotides, which are targeted to a nucleic acid encoding TGF-ß, and which modulate the expression of TGF-ß. Pharmaceutical and other compositions comprising the antisense compounds of the invention are also provided. Further provided are methods of modulating the expression of TGF-ß in cells or tissues comprising contacting said cells or tissues with one or more of the antisense compounds or compositions of the invention. Further provided are methods of treating an animal, particularly a human, suspected of having or being prone to a disease or condition associated with expression of TGF-ß by administering a therapeutically or prophylactically effective amount of one or more of the antisense compounds or compositions of the invention.

One embodiment of the present invention is a compound 8 to 50 nucleobases in length targeted to a nucleic acid molecule encoding TGF-β2 which comprises at least an 8 nucleobase portion of SEQ ID NO: 53, 54, 55, 56, 58, 60, 61, 62, 63, 64, 65 or 66 and which modulates the expression of TGF-β2. Preferably, the compound is an antisense oligonucleotide. In one aspect of this preferred embodiment, the antisense oligonucleotide comprises at lest one modified internucleoside linkage. Advantageously, the modified internucleoside linkage is a phosphorothioate linkage. Preferably, the antisense oligonucleotide comprises at least one modified sugar moiety. In one aspect of this preferred

embodiment, the modified sugar moiety is a 2'-O-methoxyethyl sugar moiety. Advantageously, the antisense oligonucleotide comprises at least one modified nucleobase. Preferably, the modified nucleobase is a 5-methylcytosine. In one aspect of this preferred embodiment, the antisense oligonucleotide is a chimeric oligonucleotide.

The present invention also provides a composition comprising the compound described above and a pharmaceutically acceptable carrier or diluent. Advantageously, the composition further comprises a colloidal dispersion system. Preferably, the compound is an antisense oligonucleotide.

Another embodiment of the present invention is a method of inhibiting the expression of TGF-β2 in cells or tissues comprising contacting the cells or tissues with the compound described above so that expression of TGF-β2 is inhibited.

The present invention also provides a method of treating an animal having a disease or condition associated with TGF-β2 comprising administering to the animal a therapeutically or prophylactically effective amount of the compound described above so that expression of TGF-β2 is inhibited. Advantageously, the disease or condition is inflammation. Preferably, the disease or condition is fibrosis or a fibrotic disease or condition. In one aspect of this preferred embodiment, the fibrotic disease or condition is fibrotic scarring, peritoneal adhesions, lung fibrosis or conjunctival scarring.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a Kaplan-Meier bleb survival curve in rabbits subjected to glaucoma drainage surgery and treatment with antisense oligonucleotides to TGF-β2, TGF-βIIR, TGF-β1 and connective tissue growth factor (CTGF).

Figure 2 is a graph showing anterior chamber depth after glaucoma surgery in rabbits after treatment with antisense oligonucleotides to TGF-β2, TGF-βIIR, TGF-β1 and CTGF.

DETAILED DESCRIPTION OF THE INVENTION

The present invention employs oligomeric antisense compounds, particularly oligonucleotides, for use in modulating the function of nucleic acid molecules encoding TGF-ß, ultimately modulating the amount of TGF-ß produced. This is accomplished by providing antisense compounds which specifically hybridize with one or more nucleic acids encoding TGF-ß. As used herein, the terms "target nucleic acid" and "nucleic acid encoding TGF-ß" encompass DNA encoding TGF-ß, RNA (including pre-mRNA and mRNA) transcribed from such DNA, and also cDNA derived from such RNA. The specific hybridization of an oligomeric compound with its target nucleic acid interferes with the normal function of the nucleic acid. This modulation of function of a target nucleic acid by compounds which specifically hybridize to it is generally referred to as "antisense". The functions of DNA to be interfered with include replication and transcription. The functions of RNA to be interfered with include all vital functions such as, for example, translocation of the RNA to the site of protein translation, translation of protein from the RNA, splicing of the RNA to yield one or more mRNA species, and catalytic activity which may be engaged in or facilitated by the RNA. The overall effect of such interference with target nucleic acid function is modulation of the expression of TGF-ß. In the context of the present invention, "modulation" means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene product. In the context of the present invention, inhibition is a preferred form of modulation of gene expression and mRNA is a preferred target. Further, since many genes (including

TGF-ß) have multiple transcripts, "modulation" also includes an alteration in the ratio between gene products, such as alteration of mRNA splice products.

It is preferred to target specific nucleic acids for antisense. "Targeting" an antisense compound to a particular nucleic acid, in the context of this invention, is a multistep process. The process usually begins with the identification of a nucleic acid sequence whose function is to be modulated. This may be, for example, a cellular gene (or mRNA transcribed from the gene) whose expression is associated with a particular disorder or disease state, or a nucleic acid molecule from an infectious agent. In the present invention, the target is a nucleic acid molecule encoding TGF-ß. The targeting process also includes determination of a site or sites within this gene for the antisense interaction to occur such that the desired effect, e.g., detection or modulation of expression of the protein, will result. Within the context of the present invention, a preferred intragenic site is the region encompassing the translation initiation or termination codon of the open reading frame (ORF) of the gene. Since, as is known in the art, the translation initiation codon is typically 5'-AUG (in transcribed mRNA molecules; 5'-ATG in the corresponding DNA molecule), the translation initiation codon is also referred to as the "AUG codon," the "start codon" or the "AUG start codon". A minority of genes have a translation initiation codon having the RNA sequence 5'-GUG, 5'-UUG or 5'-CUG, and 5'-AUA, 5'-ACG and 5'-CUG have been shown to function *in vivo*. Thus, the terms "translation initiation codon" and "start codon" can encompass many codon sequences, even though the initiator amino acid in each instance is typically methionine (in eukaryotes) or formylmethionine (in prokaryotes). It is also known in the art that eukaryotic and prokaryotic genes may have two or more alternative start codons, any one of which may be preferentially utilized for

translation initiation in a particular cell type or tissue, or under a particular set of conditions. In the context of the invention, "start codon" and "translation initiation codon" refer to the codon or codons that are used *in vivo* to initiate translation of an mRNA molecule transcribed from a gene encoding TGF-ß, regardless of the sequence(s) of such codons.

It is also known in the art that a translation termination codon (or "stop codon") of a gene may have one of three sequences, i.e., 5'-UAA, 5'-UAG and 5'-UGA (the corresponding DNA sequences are 5'-TAA, 5'-TAG and 5'-TGA, respectively). The terms "start codon region" and "translation initiation codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation initiation codon. Similarly, the terms "stop codon region" and "translation termination codon region" refer to a portion of such an mRNA or gene that encompasses from about 25 to about 50 contiguous nucleotides in either direction (i.e., 5' or 3') from a translation termination codon.

The open reading frame (ORF) or "coding region," which is known in the art to refer to the region between the translation initiation codon and the translation termination codon, is also a region which may be targeted effectively. Other target regions include the 5' untranslated region (5'UTR), known in the art to refer to the portion of an mRNA in the 5' direction from the translation initiation codon, and thus including nucleotides between the 5' cap site and the translation initiation codon of an mRNA or corresponding nucleotides on the gene, and the 3' untranslated region (3'UTR), known in the art to refer to the portion of an mRNA in the 3' direction from the translation termination codon, and thus including nucleotides between the translation termination codon and 3' end of an mRNA or corresponding

nucleotides on the gene. The 5' cap of an mRNA comprises an N7-methylated guanosine residue joined to the 5'-most residue of the mRNA via a 5'-5' triphosphate linkage. The 5' cap region of an mRNA is considered to include the 5' cap structure itself as well as the first 50 nucleotides adjacent to the cap. The 5' cap region may also be a preferred target region.

Although some eukaryotic mRNA transcripts are directly translated, many contain one or more regions, known as "introns," which are excised from a transcript before it is translated. The remaining (and therefore translated) regions are known as "exons" and are spliced together to form a continuous mRNA sequence. mRNA splice sites, i.e., intron-exon junctions, may also be preferred target regions, and are particularly useful in situations where aberrant splicing is implicated in disease, or where an overproduction of a particular mRNA splice product is implicated in disease. Aberrant fusion junctions due to rearrangements or deletions are also preferred targets. It has also been found that introns can also be effective, and therefore preferred, target regions for antisense compounds targeted, for example, to DNA or pre-mRNA.

Once one or more target sites have been identified, oligonucleotides are chosen which are sufficiently complementary to the target, i.e., hybridize sufficiently well and with sufficient specificity, to give the desired effect.

In the context of this invention, "hybridization" means hydrogen bonding, which may be Watson-Crick, Hoogsteen or reversed Hoogsteen hydrogen bonding, between complementary nucleoside or nucleotide bases. For example, adenine and thymine are complementary nucleobases which pair through the formation of hydrogen bonds. "Complementary," as used herein, refers to the capacity for precise pairing between two nucleotides. For example, if a nucleotide at a certain

position of an oligonucleotide is capable of hydrogen bonding with a nucleotide at the same position of a DNA or RNA molecule, then the oligonucleotide and the DNA or RNA are considered to be complementary to each other at that position. The oligonucleotide and the DNA or RNA are complementary to each other when a sufficient number of corresponding positions in each molecule are occupied by nucleotides which can hydrogen bond with each other. Thus, "specifically hybridizable" and "complementary" are terms which are used to indicate a sufficient degree of complementarity or precise pairing such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target. It is understood in the art that the sequence of an antisense compound need not be 100% complementary to that of its target nucleic acid to be specifically hybridizable. An antisense compound is specifically hybridizable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-target sequences under conditions in which specific binding is desired, i.e., under physiological conditions in the case of *in vivo* assays or therapeutic treatment, or in the case of *in vitro* assays, under conditions in which the assays are performed.

Antisense compounds are commonly used as research reagents and diagnostics. For example, antisense oligonucleotides, which are able to inhibit gene expression with exquisite specificity, are often used by those of ordinary skill to elucidate the function of particular genes. Antisense compounds are also used, for example, to distinguish between functions of various members of a biological pathway. Antisense modulation has, therefore, been harnessed for research use.

The specificity and sensitivity of antisense is also harnessed by those of skill in the art for therapeutic uses. Antisense oligonucleotides have been employed as therapeutic moieties in the treatment of disease states in animals and man. Antisense oligonucleotides have been safely and effectively administered to humans and numerous clinical trials are presently underway. It is thus established that oligonucleotides can be useful therapeutic modalities that can be configured to be useful in treatment regimes of cells, tissues and animals, especially humans. In the context of this invention, the term "oligonucleotide" refers to an oligomer or polymer of ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) or mimetics thereof. This term includes oligonucleotides composed of naturally-occurring nucleobases, sugars and covalent internucleoside (backbone) linkages as well as oligonucleotides having non-naturally-occurring portions which function similarly. Such modified or substituted oligonucleotides are often preferred over native forms because of desirable properties such as, for example, enhanced cellular uptake, enhanced affinity for nucleic acid target and increased stability in the presence of nucleases.

While antisense oligonucleotides are a preferred form of antisense compound, the present invention comprehends other oligomeric antisense compounds, including but not limited to oligonucleotide mimetics such as are described below. The antisense compounds in accordance with this invention preferably comprise from about 8 to about 30 nucleobases. Particularly preferred are antisense oligonucleotides comprising from about 8 to about 30 nucleobases (i.e. from about 8 to about 30 linked nucleosides). As is known in the art, a nucleoside is a base-sugar combination. The base portion of the nucleoside is normally a heterocyclic base. The two most common classes of such heterocyclic bases are the purines and the pyrimidines. Nucleotides are nucleosides that

further include a phosphate group covalently linked to the sugar portion of the nucleoside. For those nucleosides that include a pentofuranosyl sugar, the phosphate group can be linked to either the 2'-, 3'- or 5'- hydroxyl moiety of the sugar. In forming oligonucleotides, the phosphate groups covalently link adjacent nucleosides to one another to form a linear polymeric compound. In turn the respective ends of this linear polymeric structure can be further joined to form a circular structure. However, open linear structures are generally preferred. Within the oligonucleotide structure, the phosphate groups are commonly referred to as forming the internucleoside backbone of the oligonucleotide. The normal linkage or backbone of RNA and DNA is a 3'-5' phosphodiester linkage.

Specific examples of preferred antisense compounds useful in this invention include oligonucleotides containing modified backbones or non-natural internucleoside linkages. As defined in this specification, oligonucleotides having modified backbones include those that retain a phosphorus atom in the backbone and those that do not have a phosphorus atom in the backbone. For the purposes of this specification, and as sometimes referenced in the art, modified oligonucleotides that do not have a phosphorus atom in their internucleoside backbone can also be considered to be oligonucleosides.

Preferred modified oligonucleotide backbones include, for example, phosphorothioates, chiral phosphorothioates, phosphorodithioates, phosphotriesters, aminoalkylphosphotri-esters, methyl and other alkyl phosphonates including 3'-alkylene phosphonates and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thiono-alkylphosphonates, thionoalkylphosphotriesters, and borano-phosphates having normal 3'-5' linkages,2'-5' linked analogs of these, and those having inverted polarity wherein the

adjacent pairs of nucleoside units are linked 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included.

Representative United States patents that teach the preparation of the above phosphorus-containing linkages include, but are not limited to, U.S. Patents 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050, each of which is herein incorporated by reference.

Preferred modified oligonucleotide backbones that do not include a phosphorus atom therein have backbones that are formed by short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH_2 component parts.

Representative United States patents that teach the preparation of the above oligonucleosides include, but are not limited to, U.S. Patents 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070;

5,663,312; 5,633,360; 5,677,437; and 5,677,439, each of which is herein incorporated by reference.

In other preferred oligonucleotide mimetics, both the sugar and the internucleoside linkage, i.e., the backbone, of the nucleotide units are replaced with novel groups. The base units are maintained for hybridization with an appropriate nucleic acid target compound. One such oligomeric compound, an oligonucleotide mimetic that has been shown to have excellent hybridization properties, is referred to as a peptide nucleic acid (PNA). In PNA compounds, the sugar-backbone of an oligonucleotide is replaced with an amide containing backbone, in particular an aminoethylglycine backbone. The nucleobases are retained and are bound directly or indirectly to aza nitrogen atoms of the amide portion of the backbone. Representative United States patents that teach the preparation of PNA compounds include, but are not limited to, U.S. Patents 5,539,082; 5,714,331; and 5,719,262, each of which is herein incorporated by reference. Further teaching of PNA compounds can be found in Nielsen et al., *Science*, **1991**, *254*, 1497-1500.

Most preferred embodiments of the invention are oligonucleotides with phosphorothioate backbones and oligonucleosides with heteroatom backbones, and in particular $-CH_2-NH-O-CH_2-$, $-CH_2-N(CH_3)-O-CH_2-$ [known as a methylene (methylimino) or MMI backbone], $-CH_2-O-N(CH_3)-CH_2-$, $-CH_2-N(CH_3)-N(CH_3)-CH_2-$ and $-O-N(CH_3)-CH_2-CH_2-$ [wherein the native phosphodiester backbone is represented as $-O-P-O-CH_2-$] of the above referenced U.S. Patent 5,489,677, and the amide backbones of the above referenced U.S. Patent 5,602,240. Also preferred are oligonucleotides having morpholino backbone structures of the above-referenced U.S. Patent 5,034,506.

Modified oligonucleotides may also contain one or more substituted sugar moieties. Preferred oligonucleotides comprise one of the following at the 2' position: OH; F; O-,

S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C_1 to C_{10} alkyl or C_2 to C_{10} alkenyl and alkynyl. Particularly preferred are $O[(CH_2)_nO]_mCH_3$, $O(CH_2)_nOCH_3$, $O(CH_2)_nNH_2$, $O(CH_2)_nCH_3$, $O(CH_2)_nONH_2$, and $O(CH_2)_nON[(CH_2)_nCH_3)]_2$, where n and m are from 1 to about 10. Other preferred oligonucleotides comprise one of the following at the 2' position: C_1 to C_{10} lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl or O-aralkyl, SH, SCH_3, OCN, Cl, Br, CN, CF_3, OCF_3, $SOCH_3$, SO_2CH_3, ONO_2, NO_2, N_3, NH_2, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. A preferred modification includes an alkoxyalkoxy group, 2'-methoxyethoxy (2'-O-$CH_2CH_2OCH_3$, also known as 2'-O-(2-methoxyethyl) or 2'-MOE) (Martin et al., *Helv. Chim. Acta*, **1995**, *78*, 486-504). A further preferred modification includes 2'-dimethylaminooxyethoxy, i.e., a $O(CH_2)_2ON(CH_3)_2$ group, also known as 2'-DMAOE.

Other preferred modifications include 2'-methoxy (2'-O-CH_3), 2'-aminopropoxy (2'-$OCH_2CH_2CH_2NH_2$) and 2'-fluoro (2'-F). Similar modifications may also be made at other positions on the oligonucleotide, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of 5' terminal nucleotide. Oligonucleotides may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. Representative United States patents that teach the preparation of such modified sugar structures include, but are not limited to, U.S. Patent 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785;

5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,0531 5,639,873; 5,646,265; 5,658,873; 5,670,633; and 5,700,920, each of which is herein incorporated by reference.

Oligonucleotides may also include nucleobase (often referred to in the art simply as "base") modifications or substitutions. As used herein, "unmodified" or "natural" nucleobases include the purine bases adenine (A) and guanine (G), and the pyrimidine bases thymine (T), cytosine (C) and uracil (U). Modified nucleobases include other synthetic and natural nucleobases such as 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Further nucleobases include those disclosed in U.S. Patent 3,687,808, those disclosed in *The Concise Encyclopedia Of Polymer Science And Engineering*, pages 858-859, Kroschwitz, J.I., ed. John Wiley & Sons, **1990**, those disclosed by Englisch et al., *Angewandte Chemie*, International Edition, **1991**, *30*, 613, and those disclosed by Sanghvi, Y.S., Crooke, S.T., and Lebleu, B. eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 289-302. Certain of these nucleobases are particularly useful for increasing the binding affinity of the oligomeric compounds of the invention. These include 5-substituted pyrimidines, 6-azapyrimidines and N-2,

N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine substitutions have been shown to increase nucleic acid duplex stability by 0.6-1.2°C (Sanghvi, Y.S., Crooke, S.T. and Lebleu, B., eds., *Antisense Research and Applications*, CRC Press, Boca Raton, **1993**, pp. 276-278) and are presently preferred base substitutions, even more particularly when combined with 2'-O-methoxyethyl sugar modifications.

Representative United States patents that teach the preparation of certain of the above noted modified nucleobases as well as other modified nucleobases include, but are not limited to, the above noted U.S. Patent 3,687,808, as well as U.S. Patents 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,681,941; and 5,750,692, each of which is herein incorporated by reference.

Another modification of the oligonucleotides of the invention involves chemically linking to the oligonucleotide one or more moieties or conjugates which enhance the activity, cellular distribution or cellular uptake of the oligonucleotide. Such moieties include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., *Proc. Natl. Acad. Sci. USA*, **1989**, *86*, 6553-6556), cholic acid (Manoharan et al., *Bioorg. Med. Chem. Let.*, **1994**, *4*, 1053-1060), a thioether, e.g., hexyl-S-tritylthiol (Manoharan et al., *Ann. N.Y. Acad. Sci.*, **1992**, *660*, 306-309; Manoharan et al., *Bioorg. Med. Chem. Let.*, **1993**, *3*, 2765-2770), a thiocholesterol (Oberhauser et al., *Nucl. Acids Res.*, **1992**, *20*, 533-538), an aliphatic chain, e.g., dodecandiol or undecyl residues (Saison-Behmoaras et al., *EMBO J.*, **1991**, *10*, 1111-1118; Kabanov et al., *FEBS Lett.*, **1990**, *259*, 327-330;

Svinarchuk et al., *Biochimie*, **1993**, *75*, 49-54), a phospholipid, e.g., di-hexadecyl-rac-glycerol or triethyl-ammonium 1,2-di-O-hexadecyl-rac-glycero-3-H-phosphonate (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654; Shea et al., *Nucl. Acids Res.*, **1990**, *18*, 3777-3783), a polyamine or a polyethylene glycol chain (Manoharan et al., *Nucleosides & Nucleotides*, **1995**, *14*, 969-973), or adamantane acetic acid (Manoharan et al., *Tetrahedron Lett.*, **1995**, *36*, 3651-3654), a palmityl moiety (Mishra et al., *Biochim. Biophys. Acta*, **1995**, *1264*, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., *J. Pharmacol. Exp. Ther.*, **1996**, *277*, 923-937.

Representative United States patents that teach the preparation of such oligonucleotide conjugates include, but are not limited to, U.S. Patents 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717, 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241, 5,391,723; 5,416,203, 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941, each of which is herein incorporated by reference.

It is not necessary for all positions in a given compound to be uniformly modified, and in fact more than one of the aforementioned modifications may be incorporated in a single compound or even at a single nucleoside within an oligonucleotide. The present invention also includes

antisense compounds which are chimeric compounds. "Chimeric" antisense compounds or "chimeras," in the context of this invention, are antisense compounds, particularly oligonucleotides, which contain two or more chemically distinct regions, each made up of at least one monomer unit, i.e., a nucleotide in the case of an oligonucleotide compound. These oligonucleotides typically contain at least one region wherein the oligonucleotide is modified so as to confer upon the oligonucleotide increased resistance to nuclease degradation, increased cellular uptake, and/or increased binding affinity for the target nucleic acid. An additional region of the oligonucleotide may serve as a substrate for enzymes capable of cleaving RNA:DNA or RNA:RNA hybrids. By way of example, RNase H is a cellular endonuclease which cleaves the RNA strand of an RNA:DNA duplex. Activation of RNase H, therefore, results in cleavage of the RNA target, thereby greatly enhancing the efficiency of oligonucleotide inhibition of gene expression. Cleavage of the RNA target can be routinely detected by gel electrophoresis and, if necessary, associated nucleic acid hybridization techniques known in the art.

Chimeric antisense compounds of the invention may be formed as composite structures of two or more oligonucleotides, modified oligonucleotides, oligonucleosides and/or oligonucleotide mimetics as described above. Such compounds have also been referred to in the art as hybrids or gapmers. Representative United States patents that teach the preparation of such hybrid structures include, but are not limited to, U.S. Patents 5,013,830; 5,149,797; 5,220,007; 5,256,775; 5,366,878; 5,403,711; 5,491,133; 5,565,350; 5,623,065; 5,652,355; 5,652,356; and 5,700,922, each of which is herein incorporated by reference.

The antisense compounds used in accordance with this invention may be conveniently and routinely made through the

well-known technique of solid phase synthesis. Equipment for such synthesis is sold by several vendors including, for example, Applied Biosystems (Foster City, CA). Any other means for such synthesis known in the art may additionally or alternatively be employed. It is well known to use similar techniques to prepare oligonucleotides such as the phosphorothioates and alkylated derivatives.

The antisense compounds of the invention are synthesized *in vitro* and do not include antisense compositions of biological origin, or genetic vector constructs designed to direct the *in vivo* synthesis of antisense molecules.

The compounds of the invention may also be admixed, encapsulated, conjugated or otherwise associated with other molecules, molecule structures or mixtures of compounds, as for example, liposomes, receptor targeted molecules, oral, rectal, topical or other formulations, for assisting in uptake, distribution and/or absorption. Representative United States patents that teach the preparation of such uptake, distribution and/or absorption assisting formulations include, but are not limited to, U.S. Patents 5,108,921; 5,354,844; 5,416,016; 5,459,127; 5,521,291; 5,543,158; 5,547,932; 5,583,020; 5,591,721; 4,426,330; 4,534,899; 5,013,556; 5,108,921; 5,213,804; 5,227,170; 5,264,221; 5,356,633; 5,395,619; 5,416,016; 5,417,978; 5,462,854; 5,469,854; 5,512,295; 5,527,528; 5,534,259; 5,543,152; 5,556,948; 5,580,575; and 5,595,756, each of which is herein incorporated by reference.

The antisense compounds of the invention encompass any pharmaceutically acceptable salts, esters, or salts of such esters, or any other compound which, upon administration to an animal including a human, is capable of providing (directly or indirectly) the biologically active metabolite or residue thereof. Accordingly, for example, the disclosure is also drawn to prodrugs and pharmaceutically acceptable salts of the

compounds of the invention, pharmaceutically acceptable salts of such prodrugs, and other bioequivalents.

The term "prodrug" indicates a therapeutic agent that is prepared in an inactive form that is converted to an active form (i.e., drug) within the body or cells thereof by the action of endogenous enzymes or other chemicals and/or conditions. In particular, prodrug versions of the oligonucleotides of the invention are prepared as SATE [(S-acetyl-2-thioethyl) phosphate] derivatives according to the methods disclosed in WO 93/24510 or in WO 94/26764.

The term "pharmaceutically acceptable salts" refers to physiologically and pharmaceutically acceptable salts of the compounds of the invention: i.e., salts that retain the desired biological activity of the parent compound and do not impart undesired toxicological effects thereto.

Pharmaceutically acceptable base addition salts are formed with metals or amines, such as alkali and alkaline earth metals or organic amines. Examples of metals used as cations are sodium, potassium, magnesium, calcium, and the like. Examples of suitable amines are N,N'-dibenzylethylenediamine, chloroprocaine, choline, diethanolamine, dicyclohexylamine, ethylenediamine, N-methylglucamine, and procaine (see, for example, Berge et al., "Pharmaceutical Salts," *J. of Pharma Sci.*, **1977**, *66,* 1-19). The base addition salts of said acidic compounds are prepared by contacting the free acid form with a sufficient amount of the desired base to produce the salt in the conventional manner. The free acid form may be regenerated by contacting the salt form with an acid and isolating the free acid in the conventional manner. The free acid forms differ from their respective salt forms somewhat in certain physical properties such as solubility in polar solvents, but otherwise the salts are equivalent to their respective free acid for purposes of the present invention. As used herein, a

"pharmaceutical addition salt" includes a pharmaceutically acceptable salt of an acid form of one of the components of the compositions of the invention. These include organic or inorganic acid salts of the amines. Preferred addition salts are acid salts such as the hydrochlorides, acetates, salicylates, nitrates and phosphates. Other suitable pharmaceutically acceptable salts are well known to those skilled in the art and include basic salts of a variety of inorganic and organic acids, such as, for example, with inorganic acids, such as for example hydrochloric acid, hydrobromic acid, sulfuric acid or phosphoric acid; with organic carboxylic, sulfonic, sulfo or phospho acids or N-substituted sulfamic acids, for example acetic acid, propionic acid, glycolic acid, succinic acid, maleic acid, hydroxymaleic acid, methylmaleic acid, fumaric acid, malic acid, tartaric acid, lactic acid, oxalic acid, gluconic acid, glucaric acid, glucuronic acid, citric acid, benzoic acid, cinnamic acid, mandelic acid, salicylic acid, 4-aminosalicylic acid, 2-phenoxybenzoic acid, 2-acetoxybenzoic acid, embolic acid, nicotinic acid or isonicotinic acid; and with amino acids, such as the 20 alpha-amino acids involved in the synthesis of proteins in nature, for example glutamic acid or aspartic acid, and also with phenylacetic acid, methanesulfonic acid, ethanesulfonic acid, 2-hydroxyethanesulfonic acid, ethane-1,2-disulfonic acid, benzenesulfonic acid, 4-methylbenzenesulfoic acid, naphthalene-2-sulfonic acid, naphthalene-1,5-disulfonic acid, 2- or 3-phosphoglycerate, glucose-6-phosphate, N-cyclohexylsulfamic acid (with the formation of cyclamates), or with other acid organic compounds, such as ascorbic acid. Pharmaceutically acceptable salts of compounds may also be prepared with a pharmaceutically acceptable cation. Suitable pharmaceutically acceptable cations are well known to those skilled in the art and include alkaline, alkaline earth,

ammonium and quaternary ammonium cations. Carbonates or hydrogen carbonates are also possible.

For oligonucleotides, preferred examples of pharmaceutically acceptable salts include but are not limited to (a) salts formed with cations such as sodium, potassium, ammonium, magnesium, calcium, polyamines such as spermine and spermidine, etc.; (b) acid addition salts formed with inorganic acids, for example hydrochloric acid, hydrobromic acid, sulfuric acid, phosphoric acid, nitric acid and the like; (c) salts formed with organic acids such as, for example, acetic acid, oxalic acid, tartaric acid, succinic acid, maleic acid, fumaric acid, gluconic acid, citric acid, malic acid, ascorbic acid, benzoic acid, tannic acid, palmitic acid, alginic acid, polyglutamic acid, naphthalenesulfonic acid, methanesulfonic acid, p-toluenesulfonic acid, naphthalenedisulfonic acid, polygalacturonic acid, and the like; and (d) salts formed from elemental anions such as chlorine, bromine, and iodine.

The antisense compounds of the present invention can be utilized for diagnostics, therapeutics, prophylaxis and as research reagents and kits. For therapeutics, an animal, preferably a human, suspected of having a disease or disorder which can be treated by modulating the expression of TGF-ß is treated by administering antisense compounds in accordance with this invention. The compounds of the invention can be utilized in pharmaceutical compositions by adding an effective amount of an antisense compound to a suitable pharmaceutically acceptable diluent or carrier. Use of the antisense compounds and methods of the invention may also be useful prophylactically, e.g., to prevent or delay infection, inflammation or tumor formation, for example.

The antisense compounds of the invention are useful for research and diagnostics, because these compounds hybridize to nucleic acids encoding TGF-ß, enabling sandwich and other

assays to easily be constructed to exploit this fact. Hybridization of the antisense oligonucleotides of the invention with a nucleic acid encoding TGF-ß can be detected by means known in the art. Such means may include conjugation of an enzyme to the oligonucleotide, radiolabelling of the oligonucleotide or any other suitable detection means. Kits using such detection means for detecting the level of TGF-ß in a sample may also be prepared.

The present invention also includes pharmaceutical compositions and formulations which include the antisense compounds of the invention. The pharmaceutical compositions of the present invention may be administered in a number of ways depending upon whether local or systemic treatment is desired and upon the area to be treated. Administration may be topical (including ophthalmic and to mucous membranes including vaginal and rectal delivery), pulmonary, e.g., by inhalation or insufflation of powders or aerosols, including by nebulizer; intratracheal, intranasal, epidermal, intradermal and transdermal), oral or parenteral. Parenteral administration includes intravenous, intraarterial, subcutaneous, intraperitoneal or intramuscular injection or infusion; or intracranial, e.g., intrathecal or intraventricular, administration. Oligonucleotides with at least one 2'-O-methoxyethyl modification are believed to be particularly useful for oral administration.

Pharmaceutical compositions and formulations for topical administration may include transdermal patches, ointments, lotions, creams, gels, drops, suppositories, sprays, liquids and powders. Conventional pharmaceutical carriers, aqueous, powder or oily bases, thickeners and the like may be necessary or desirable. Coated condoms, gloves and the like may also be useful.

Compositions and formulations for oral administration include powders or granules, suspensions or solutions in water or non-aqueous media, capsules, sachets or tablets. Thickeners, flavoring agents, diluents, emulsifiers, dispersing aids or binders may be desirable.

Compositions and formulations for parenteral, intrathecal or intraventricular administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives such as, but not limited to, penetration enhancers, carrier compounds and other pharmaceutically acceptable carriers or excipients.

Pharmaceutical compositions and/or formulations comprising the oligonucleotides of the present invention may also include penetration enhancers in order to enhance the alimentary delivery of the oligonucleotides. Penetration enhancers may be classified as belonging to one of five broad categories, i.e., fatty acids, bile salts, chelating agents, surfactants and non-surfactants (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, *8*, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7, 1-33). One or more penetration enhancers from one or more of these broad categories may be included.

Various fatty acids and their derivatives which act as penetration enhancers include, for example, oleic acid, lauric acid, capric acid, myristic acid, palmitic acid, stearic acid, linoleic acid, linolenic acid, dicaprate, tricaprate, recinleate, monoolein (a.k.a. 1-monooleoyl-rac-glycerol), dilaurin, caprylic acid, arichidonic acid, glyceryl 1-monocaprate, 1-dodecylazacycloheptan-2-one, acylcarnitines, acylcholines, mono- and di-glycerides and physiologically acceptable salts thereof (i.e., oleate, laurate, caprate, myristate, palmitate, stearate, linoleate, etc.) (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, *8*:2, 91-192; Muranishi, *Critical Reviews in Therapeutic Drug*

Carrier Systems, **1990**, 7:1, 1-33; El-Hariri et al., *J. Pharm. Pharmacol.*, **1992**, *44*, 651-654). Examples of some presently preferred fatty acids are sodium caprate and sodium laurate, used singly or in combination at concentrations of 0.5 to 5%.

The physiological roles of bile include the facilitation of dispersion and absorption of lipids and fat-soluble vitamins (Brunton, Chapter 38 In: *Goodman & Gilman's The Pharmacological Basis of Therapeutics*, 9th Ed., Hardman et al., eds., McGraw-Hill, New York, NY, **1996**, pages 934-935). Various natural bile salts, and their synthetic derivatives, act as penetration enhancers. Thus, the term "bile salt" includes any of the naturally occurring components of bile as well as any of their synthetic derivatives. A presently preferred bile salt is chenodeoxycholic acid (CDCA) (Sigma Chemical Company, St. Louis, MO), generally used at concentrations of 0.5 to 2%.

Complex formulations comprising one or more penetration enhancers may be used. For example, bile salts may be used in combination with fatty acids to make complex formulations. Preferred combinations include CDCA combined with sodium caprate or sodium laurate (generally 0.5 to 5%).

Chelating agents include, but are not limited to, disodium ethylenediaminetetraacetate (EDTA), citric acid, salicylates (e.g., sodium salicylate, 5-methoxysalicylate and homovanilate), N-acyl derivatives of collagen, laureth-9 and N-amino acyl derivatives of beta-diketones (enamines)(Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, *8*:2, 92-192; Muranishi, *Critical Reviews in Therapeutic Drug Carrier Systems*, **1990**, 7:1, 1-33; Buur et al., *J. Control Rel.*, **1990**, *14*, 43-51). Chelating agents have the added advantage of also serving as DNase inhibitors.

Surfactants include, for example, sodium lauryl sulfate, polyoxyethylene-9-lauryl ether and polyoxyethylene-20-cetyl

ether (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, *8*:2, 92-191); and perfluorochemical emulsions, such as FC-43 (Takahashi et al., *J. Pharm. Pharmacol.*, **1988**, *40*, 252-257).

Non-surfactants include, for example, unsaturated cyclic ureas, 1-alkyl- and 1-alkenylazacyclo-alkanone derivatives (Lee et al., *Critical Reviews in Therapeutic Drug Carrier Systems*, **1991**, *8*:2, 92-191); and non-steroidal anti-inflammatory agents such as diclofenac sodium, indomethacin and phenylbutazone (Yamashita et al., *J. Pharm. Pharmacol.*, **1987**, *39*, 621-626).

As used herein, "carrier compound" refers to a nucleic acid, or analog thereof, which is inert (i.e., does not possess biological activity per se) but is recognized as a nucleic acid by *in vivo* processes that reduce the bioavailability of a nucleic acid having biological activity by, for example, degrading the biologically active nucleic acid or promoting its removal from circulation. The coadministration of a nucleic acid and a carrier compound, typically with an excess of the latter substance, can result in a substantial reduction of the amount of nucleic acid recovered in the liver, kidney or other extracirculatory reservoirs, presumably due to competition between the carrier compound and the nucleic acid for a common receptor. For example, the recovery of a partially phosphorothioated oligonucleotide in hepatic tissue is reduced when it is coadministered with polyinosinic acid, dextran sulfate, polycytidic acid or 4-acetamido-4'-isothiocyano-stilbene-2,2'-disulfonic acid (Miyao et al., *Antisense Res. Dev.*, **1995**, *5*, 115-121; Takakura et al., *Antisense & Nucl. Acid Drug Dev.*, **1996**, *6*, 177-183).

In contrast to a carrier compound, a "pharmaceutically acceptable carrier" (excipient) is a pharmaceutically

acceptable solvent, suspending agent or any other pharmacologically inert vehicle for delivering one or more nucleic acids to an animal. The pharmaceutically acceptable carrier may be liquid or solid and is selected with the planned manner of administration in mind so as to provide for the desired bulk, consistency, etc., when combined with a nucleic acid and the other components of a given pharmaceutical composition. Typical pharmaceutically acceptable carriers include, but are not limited to, binding agents (e.g., pregelatinized maize starch, polyvinyl-pyrrolidone or hydroxypropyl methylcellulose, etc.); fillers (e.g., lactose and other sugars, microcrystalline cellulose, pectin, gelatin, calcium sulfate, ethyl cellulose, polyacrylates or calcium hydrogen phosphate, etc.); lubricants (e.g., magnesium stearate, talc, silica, colloidal silicon dioxide, stearic acid, metallic stearates, hydrogenated vegetable oils, corn starch, polyethylene glycols, sodium benzoate, sodium acetate, etc.); disintegrates (e.g., starch, sodium starch glycolate, etc.); or wetting agents (e.g., sodium lauryl sulphate, etc.). Sustained release oral delivery systems and/or enteric coatings for orally administered dosage forms are described in U.S. Patents 4,704,295; 4,556,552; 4,309,406; and 4,309,404.

The compositions of the present invention may additionally contain other adjunct components conventionally found in pharmaceutical compositions, at their art-established usage levels. Thus, for example, the compositions may contain additional compatible pharmaceutically-active materials such as, e.g., antipruritics, astringents, local anesthetics or anti-inflammatory agents, or may contain additional materials useful in physically formulating various dosage forms of the composition of present invention, such as dyes, flavoring agents, preservatives, antioxidants, opacifiers, thickening agents and stabilizers. However, such materials, when added,

should not unduly interfere with the biological activities of the components of the compositions of the invention.

Regardless of the method by which the antisense compounds of the invention are introduced into a patient, colloidal dispersion systems may be used as delivery vehicles to enhance the *in vivo* stability of the compounds and/or to target the compounds to a particular organ, tissue or cell type. Colloidal dispersion systems include, but are not limited to, macromolecule complexes, nanocapsules, microspheres, beads and lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, liposomes and lipid:oligonucleotide complexes of uncharacterized structure. A preferred colloidal dispersion system is a plurality of liposomes. Liposomes are microscopic spheres having an aqueous core surrounded by one or more outer layer(s) made up of lipids arranged in a bilayer configuration (see, generally, Chonn et al., *Current Op. Biotech.*, **1995**, *6*, 698-708).

Certain embodiments of the invention provide for liposomes and other compositions containing (a) one or more antisense compounds and (b) one or more other chemotherapeutic agents which function by a non-antisense mechanism. Examples of such chemotherapeutic agents include, but are not limited to, anticancer drugs such as daunorubicin, dactinomycin, doxorubicin, bleomycin, mitomycin, nitrogen mustard, chlorambucil, melphalan, cyclophosphamide, 6-mercaptopurine, 6-thioguanine, cytarabine (CA), 5-fluorouracil (5-FU), floxuridine (5-FUdR), methotrexate (MTX), colchicine, vincristine, vinblastine, etoposide, teniposide, cisplatin and diethylstilbestrol (DES). See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pp. 1206-1228. Anti-inflammatory drugs, including but not limited to nonsteroidal anti-inflammatory drugs and corticosteroids, and antiviral drugs, including but not limited to ribivirin, vidarabine, acyclovir and

ganciclovir, may also be combined in compositions of the invention. See, generally, *The Merck Manual of Diagnosis and Therapy*, 15th Ed., Berkow et al., eds., **1987**, Rahway, N.J., pp. 2499-2506 and 46-49, respectively. Other non-antisense chemotherapeutic agents are also within the scope of this invention. Two or more combined compounds may be used together or sequentially.

In another related embodiment, compositions of the invention may contain one or more antisense compounds, particularly oligonucleotides, targeted to a first nucleic acid and one or more additional antisense compounds targeted to a second nucleic acid target. Two or more combined compounds may be used together or sequentially.

The formulation of therapeutic compositions and their subsequent administration is believed to be within the skill of those in the art. Dosing is dependent on severity and responsiveness of the disease state to be treated, with the course of treatment lasting from several days to several months, or until a cure is effected or a diminution of the disease state is achieved. Optimal dosing schedules can be calculated from measurements of drug accumulation in the body of the patient. Persons of ordinary skill can easily determine optimum dosages, dosing methodologies and repetition rates. Optimum dosages may vary depending on the relative potency of individual oligonucleotides, and can generally be estimated based on EC_{50}s found to be effective in *in vitro* and *in vivo* animal models. In general, dosage is from 0.01 μg to 100 g per kg of body weight, and may be given once or more daily, weekly, monthly or yearly, or even once every 2 to 20 years. Persons of ordinary skill in the art can easily estimate repetition rates for dosing based on measured residence times and concentrations of the drug in bodily fluids or tissues. Following successful treatment, it may be desirable to have the patient undergo maintenance therapy to

prevent the recurrence of the disease state, wherein the oligonucleotide is administered in maintenance doses, ranging from 0.01 μg to 100 g per kg of body weight, once or more daily, to once every 20 years.

While the present invention has been described with specificity in accordance with certain of its preferred embodiments, the following examples serve only to illustrate the invention and are not intended to limit the same.

EXAMPLES

Example 1

Nucleoside Phosphoramidites For Oligonucleotide Synthesis Deoxy And 2'-Alkoxy Amidites

2'-Deoxy and 2'-methoxy beta-cyanoethyldiisopropyl phosphoramidites were purchased from commercial sources (e.g. Chemgenes, Needham MA or Glen Research, Inc. Sterling VA). Other 2'-O-alkoxy substituted nucleoside amidites are prepared as described in U.S. Patent 5,506,351, herein incorporated by reference. For oligonucleotides synthesized using 2'-alkoxy amidites, the standard cycle for unmodified oligonucleotides was utilized, except the wait step after pulse delivery of tetrazole and base was increased to 360 seconds. Oligonucleotides containing 5-methyl-2'-deoxycytidine (5-Me-C) nucleotides were synthesized according to published methods (Sanghvi, et. al., *Nucleic Acids Research*, **1993**, *21*, 3197-3203] using commercially available phosphoramidites (Glen Research, Sterling VA or ChemGenes, Needham MA).

2'-Fluoro amidites

2'-Fluorodeoxyadenosine amidites

2'-fluoro oligonucleotides were synthesized as described previously by Kawasaki, et. al., *J. Med. Chem.*, **1993**, *36*, 831-841 and U.S. Patent 5,670,633, herein incorporated by

reference. Briefly, the protected nucleoside N6-benzoyl-2'-deoxy-2'-fluoroadenosine was synthesized utilizing commercially available 9-beta-D-arabinofuranosyladenine as starting material and by modifying literature procedures whereby the 2'-alpha-fluoro atom is introduced by a S_N2-displacement of a 2'-beta-trityl group. Thus N6-benzoyl-9-beta-D-arabinofuranosyladenine was selectively protected in moderate yield as the 3',5'-ditetrahydropyranyl (THP) intermediate. Deprotection of the THP and N6-benzoyl groups was accomplished using standard methodologies and standard methods were used to obtain the 5'-dimethoxytrityl-(DMT) and 5'-DMT-3'-phosphoramidite intermediates.

2'-Fluorodeoxyguanosine

The synthesis of 2'-deoxy-2'-fluoroguanosine was accomplished using tetraisopropyldisiloxanyl (TPDS) protected 9-beta-D-arabinofuranosylguanine as starting material, and conversion to the intermediate diisobutyryl-arabinofuranosylguanosine. Deprotection of the TPDS group was followed by protection of the hydroxyl group with THP to give diisobutyryl di-THP protected arabinofuranosylguanine. Selective O-deacylation and triflation was followed by treatment of the crude product with fluoride, then deprotection of the THP groups. Standard methodologies were used to obtain the 5'-DMT- and 5'-DMT-3'-phosphoramidites.

2'-Fluorouridine

Synthesis of 2'-deoxy-2'-fluorouridine was accomplished by the modification of a literature procedure in which 2,2'-anhydro-1-beta-D-arabinofuranosyluracil was treated with 70% hydrogen fluoride-pyridine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-Fluorodeoxycytidine

2'-deoxy-2'-fluorocytidine was synthesized via amination of 2'-deoxy-2'-fluorouridine, followed by selective protection to give N4-benzoyl-2'-deoxy-2'-fluorocytidine. Standard procedures were used to obtain the 5'-DMT and 5'-DMT-3'phosphoramidites.

2'-O-(2-Methoxyethyl) modified amidites

2'-O-Methoxyethyl-substituted nucleoside amidites are prepared as follows, or alternatively, as per the methods of Martin, P., *Helvetica Chimica Acta*, **1995**, *78*, 486-504.

2,2'-Anhydro[1-(beta-D-arabinofuranosyl)-5-methyluridine]

5-Methyluridine (ribosylthymine, commercially available through Yamasa, Choshi, Japan) (72.0 g, 0.279 M), diphenyl-carbonate (90.0 g, 0.420 M) and sodium bicarbonate (2.0 g, 0.024 M) were added to DMF (300 mL). The mixture was heated to reflux, with stirring, allowing the evolved carbon dioxide gas to be released in a controlled manner. After 1 hour, the slightly darkened solution was concentrated under reduced pressure. The resulting syrup was poured into diethylether (2.5 L), with stirring. The product formed a gum. The ether was decanted and the residue was dissolved in a minimum amount of methanol (ca. 400 mL). The solution was poured into fresh ether (2.5 L) to yield a stiff gum. The ether was decanted and the gum was dried in a vacuum oven (60°C at 1 mm Hg for 24 hours) to give a solid that was crushed to a light tan powder (57 g, 85% crude yield). The NMR spectrum was consistent with the structure, contaminated with phenol as its sodium salt (ca. 5%). The material was used as is for further reactions or purified further by column chromatography using a gradient

of methanol in ethyl acetate (10-25%) to give a white solid, mp 222-4°C.

2'-O-Methoxyethyl-5-methyluridine

2,2'-Anhydro-5-methyluridine (195 g, 0.81 M), tris(2-methoxyethyl)borate (231 g, 0.98 M) and 2-methoxyethanol (1.2 L) were added to a 2 L stainless steel pressure vessel and placed in a pre-heated oil bath at 160°C. After heating for 48 hours at 155-160°C, the vessel was opened and the solution evaporated to dryness and triturated with MeOH (200 mL). The residue was suspended in hot acetone (1 L). The insoluble salts were filtered, washed with acetone (150 mL) and the filtrate evaporated. The residue (280 g) was dissolved in CH_3CN (600 mL) and evaporated. A silica gel column (3 kg) was packed in CH_2Cl_2/Acetone/MeOH (20:5:3) containing 0.5% Et_3NH. The residue was dissolved in CH_2Cl_2 (250 mL) and adsorbed onto silica (150 g) prior to loading onto the column. The product was eluted with the packing solvent to give 160 g (63%) of product. Additional material was obtained by reworking impure fractions.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5-methyluridine (160 g, 0.506 M) was co-evaporated with pyridine (250 mL) and the dried residue dissolved in pyridine (1.3 L). A first aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the mixture stirred at room temperature for one hour. A second aliquot of dimethoxytrityl chloride (94.3 g, 0.278 M) was added and the reaction stirred for an additional one hour. Methanol (170 mL) was then added to stop the reaction. HPLC showed the presence of approximately 70% product. The solvent was evaporated and triturated with CH_3CN (200 mL). The residue was dissolved in $CHCl_3$ (1.5 L) and extracted with 2x500 mL of saturated $NaHCO_3$ and 2x500 mL of saturated NaCl.

The organic phase was dried over Na_2SO_4, filtered and evaporated. 275 g of residue was obtained. The residue was purified on a 3.5 kg silica gel column, packed and eluted with EtOAc/Hexane/Acetone (5:5:1) containing 0.5% Et_3NH. The pure fractions were evaporated to give 164 g of product. Approximately 20 g additional was obtained from the impure fractions to give a total yield of 183 g (57%).

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (106 g, 0.167 M), DMF/pyridine (750 mL of a 3:1 mixture prepared from 562 mL of DMF and 188 mL of pyridine) and acetic anhydride (24.38 mL, 0.258 M) were combined and stirred at room temperature for 24 hours. The reaction was monitored by tlc by first quenching the tlc sample with the addition of MeOH. Upon completion of the reaction, as judged by tlc, MeOH (50 mL) was added and the mixture evaporated at 35°C. The residue was dissolved in $CHCl_3$ (800 mL) and extracted with 2x200 mL of saturated sodium bicarbonate and 2x200 mL of saturated NaCl. The water layers were back extracted with 200 mL of $CHCl_3$. The combined organics were dried with sodium sulfate and evaporated to give 122 g of residue (approx. 90% product). The residue was purified on a 3.5 kg silica gel column and eluted using EtOAc/Hexane(4:1). Pure product fractions were evaporated to yield 96 g (84%). An additional 1.5 g was recovered from later fractions.

3'-O-Acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine

A first solution was prepared by dissolving 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyluridine (96 g, 0.144 M) in CH_3CN (700 mL) and set aside. Triethylamine (189 mL, 1.44 M) was added to a solution of triazole (90 g, 1.3 M)

in CH_3CN (1 L), cooled to -5°C and stirred for 0.5 hours using an overhead stirrer. $POCl_3$ was added dropwise, over a 30 minute period, to the stirred solution maintained at 0-10°C, and the resulting mixture stirred for an additional 2 hours. The first solution was added dropwise, over a 45 minute period, to the latter solution. The resulting reaction mixture was stored overnight in a cold room. Salts were filtered from the reaction mixture and the solution was evaporated. The residue was dissolved in EtOAc (1 L) and the insoluble solids were removed by filtration. The filtrate was washed with 1x300 mL of $NaHCO_3$ and 2x300 mL of saturated NaCl, dried over sodium sulfate and evaporated. The residue was triturated with EtOAc to give the title compound.

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

A solution of 3'-O-acetyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methyl-4-triazoleuridine (103 g, 0.141 M) in dioxane (500 mL) and NH_4OH (30 mL) was stirred at room temperature for 2 hours. The dioxane solution was evaporated and the residue azeotroped with MeOH (2x200 mL). The residue was dissolved in MeOH (300 mL) and transferred to a 2 liter stainless steel pressure vessel. MeOH (400 mL) saturated with NH_3 gas was added and the vessel heated to 100°C for 2 hours (tlc showed complete conversion). The vessel contents were evaporated to dryness and the residue was dissolved in EtOAc (500 mL) and washed once with saturated NaCl (200 mL). The organics were dried over sodium sulfate and the solvent was evaporated to give 85 g (95%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine

2'-O-Methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (85 g, 0.134 M) was dissolved in DMF (800 mL) and benzoic anhydride (37.2 g, 0.165 M) was added with stirring. After stirring for 3 hours, tlc showed the reaction to be approximately 95% complete. The solvent was evaporated and the residue azeotroped with MeOH (200 mL). The residue was dissolved in $CHCl_3$ (700 mL) and extracted with saturated $NaHCO_3$ (2x300 mL) and saturated NaCl (2x300 mL), dried over $MgSO_4$ and evaporated to give a residue (96 g). The residue was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (1:1) containing 0.5% Et_3NH as the eluting solvent. The pure product fractions were evaporated to give 90 g (90%) of the title compound.

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine-3'-amidite

N4-Benzoyl-2'-O-methoxyethyl-5'-O-dimethoxytrityl-5-methylcytidine (74 g, 0.10 M) was dissolved in CH_2Cl_2 (1 L). Tetrazole diisopropylamine (7.1 g) and 2-cyanoethoxy-tetra-(isopropyl)phosphite (40.5 mL, 0.123 M) were added with stirring, under a nitrogen atmosphere. The resulting mixture was stirred for 20 hours at room temperature (tlc showed the reaction to be 95% complete). The reaction mixture was extracted with saturated $NaHCO_3$ (1x300 mL) and saturated NaCl (3x300 mL). The aqueous washes were back-extracted with CH_2Cl_2 (300 mL), and the extracts were combined, dried over $MgSO_4$ and concentrated. The residue obtained was chromatographed on a 1.5 kg silica column using EtOAc/Hexane (3:1) as the eluting solvent. The pure fractions were combined to give 90.6 g (87%) of the title compound.

Example 2

Oligonucleotide Synthesis

Unsubstituted and substituted phosphodiester (P=O) oligonucleotides are synthesized on an automated DNA synthesizer (Applied Biosystems model 380B) using standard phosphoramidite chemistry with oxidation by iodine.

Phosphorothioates (P=S) are synthesized as per the phosphodiester oligonucleotides except the standard oxidation bottle was replaced by 0.2 M solution of 3H-1,2-benzodithiole-3-one 1,1-dioxide in acetonitrile for the stepwise thiation of the phosphite linkages. The thiation wait step was increased to 68 seconds and was followed by the capping step. After cleavage from the CPG column and deblocking in concentrated ammonium hydroxide at 55°C (18 hr), the oligonucleotides were purified by precipitating twice with 2.5 volumes of ethanol from a 0.5 M NaCl solution.

Phosphinate oligonucleotides are prepared as described in U.S. Patent 5,508,270, herein incorporated by reference.

Alkyl phosphonate oligonucleotides are prepared as described in U.S. Patent 4,469,863, herein incorporated by reference.

3'-Deoxy-3'-methylene phosphonate oligonucleotides are prepared as described in U.S. Patents 5,610,289 or 5,625,050, herein incorporated by reference.

Phosphoramidite oligonucleotides are prepared as described in U.S. Patent 5,256,775 or U.S. Patent 5,366,878, herein incorporated by reference.

Alkylphosphonothioate oligonucleotides are prepared as described in published PCT applications PCT/US94/00902 and PCT/US93/06976 (published as WO 94/17093 and WO 94/02499, respectively), herein incorporated by reference.

3'-Deoxy-3'-amino phosphoramidate oligonucleotides are prepared as described in U.S. Patent 5,476,925, herein incorporated by reference.

Phosphotriester oligonucleotides are prepared as described in U.S. Patent 5,023,243, herein incorporated by reference.

Borano phosphate oligonucleotides are prepared as described in U.S. Patents 5,130,302 and 5,177,198, both herein incorporated by reference.

Example 3

Oligonucleoside Synthesis

Methylenemethylimino linked oligonucleosides, also identified as MMI linked oligonucleosides, methylenedimethyl-hydrazo linked oligonucleosides, also identified as MDH linked oligonucleosides, and methylenecarbonylamino linked oligonucleosides, also identified as amide-3 linked oligonucleosides, and methyleneaminocarbonyl linked oligo-nucleosides, also identified as amide-4 linked oligonucleo-sides, as well as mixed backbone compounds having, for instance, alternating MMI and P=O or P=S linkages are prepared as described in U.S. Patents 5,378,825, 5,386,023, 5,489,677, 5,602,240 and 5,610,289, all of which are herein incorporated by reference.

Formacetal and thioformacetal linked oligonucleosides are prepared as described in U.S. Patents 5,264,562 and 5,264,564, herein incorporated by reference.

Ethylene oxide linked oligonucleosides are prepared as described in U.S. Patent 5,223,618, herein incorporated by reference.

Example 4

PNA Synthesis

Peptide nucleic acids (PNAs) are prepared in accordance with any of the various procedures referred to in Peptide Nucleic Acids (PNA): Synthesis, Properties and Potential Applications, *Bioorganic & Medicinal Chemistry*, **1996**, *4*, 5-23.

They may also be prepared in accordance with U.S. Patents 5,539,082, 5,700,922, and 5,719,262, herein incorporated by reference.

Example 5

Synthesis of Chimeric Oligonucleotides

Chimeric oligonucleotides, oligonucleosides or mixed oligonucleotides/oligonucleosides of the invention can be of several different types. These include a first type wherein the "gap" segment of linked nucleosides is positioned between 5' and 3' "wing" segments of linked nucleosides and a second "open end" type wherein the "gap" segment is located at either the 3' or the 5' terminus of the oligomeric compound. Oligonucleotides of the first type are also known in the art as "gapmers" or gapped oligonucleotides. Oligonucleotides of the second type are also known in the art as "hemimers" or "wingmers".

[2'-O-Me]--[2'-deoxy]--[2'-O-Me]Chimeric Phosphorothioate Oligonucleotides

Chimeric oligonucleotides having 2'-O-alkyl phosphorothioate and 2'-deoxy phosphorothioate oligonucleotide segments are synthesized using an Applied Biosystems automated DNA synthesizer Model 380B, as above. Oligonucleotides are synthesized using the automated synthesizer and 2'-deoxy-5'-dimethoxytrityl-3'-O-phosphoramidite for the DNA portion and 5'-dimethoxytrityl-2'-O-methyl-3'-O-phosphoramidite for 5' and 3' wings. The standard synthesis cycle is modified by increasing the wait step after the delivery of tetrazole and base to 600 s repeated four times for RNA and twice for 2'-O-methyl. The fully protected oligonucleotide is cleaved from the support and the phosphate group is deprotected in 3:1 Ammonia/Ethanol at room temperature overnight then lyophilized to dryness. Treatment in methanolic ammonia for 24 hours at

room temperature is then done to deprotect all bases and sample was again lyophilized to dryness. The pellet is resuspended in 1M TBAF in THF for 24 hours at room temperature to deprotect the 2' positions. The reaction is then quenched with 1M TEAA and the sample is then reduced to 1/2 volume by rotovac before being desalted on a G25 size exclusion column. The oligo recovered is then analyzed spectrophotometrically for yield and for purity by capillary electrophoresis and by mass spectrometry.

[2'-O-(2-Methoxyethyl)]--[2'-deoxy]--[2'-O-(Methoxyethyl)] Chimeric Phosphorothioate Oligonucleotides

[2'-O-(2-methoxyethyl)]--[2'-deoxy]--[-2'-O-(methoxyethyl)] chimeric phosphorothioate oligonucleotides were prepared as per the procedure above for the 2'-O-methyl chimeric oligonucleotide, with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites.

[2'-O-(2-Methoxyethyl)Phosphodiester]--[2'-deoxy Phosphorothioate]--[2'-O-(2-Methoxyethyl) Phosphodiester] Chimeric Oligonucleotides

[2'-O-(2-methoxyethyl phosphodiester]--[2'-deoxy phosphorothioate]--[2'-O-(methoxyethyl) phosphodiester] chimeric oligonucleotides are prepared as per the above procedure for the 2'-O-methyl chimeric oligonucleotide with the substitution of 2'-O-(methoxyethyl) amidites for the 2'-O-methyl amidites, oxidization with iodine to generate the phosphodiester internucleotide linkages within the wing portions of the chimeric structures and sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) to generate the phosphorothioate internucleotide linkages for the center gap.

Other chimeric oligonucleotides, chimeric oligonucleosides and mixed chimeric oligonucleotides/oligonucleosides are synthesized according to U.S. Patent 5,623,065, herein incorporated by reference.

Example 6

Oligonucleotide Isolation

After cleavage from the controlled pore glass column (Applied Biosystems) and deblocking in concentrated ammonium hydroxide at 55°C for 18 hours, the oligonucleotides or oligonucleosides were purified by precipitation twice out of 0.5 M NaCl with 2.5 volumes ethanol. Synthesized oligonucleotides were analyzed by polyacrylamide gel electrophoresis on denaturing gels and judged to be at least 85% full length material. The relative amounts of phosphorothioate and phosphodiester linkages obtained in synthesis were periodically checked by ^{31}P nuclear magnetic resonance spectroscopy, and for some studies oligonucleotides were purified by HPLC, as described by Chiang et al., *J. Biol. Chem.* **1991**, *266*, 18162-18171. Results obtained with HPLC-purified material were similar to those obtained with non-HPLC purified material.

Example 7

Oligonucleotide Synthesis - 96 Well Plate Format

Oligonucleotides are synthesized via solid phase P(III) phosphoramidite chemistry on an automated synthesizer capable of assembling 96 sequences simultaneously in a standard 96 well format. Phosphodiester internucleotide linkages are afforded by oxidation with aqueous iodine. Phosphorothioate internucleotide linkages are generated by sulfurization utilizing 3,H-1,2 benzodithiole-3-one 1,1 dioxide (Beaucage Reagent) in anhydrous acetonitrile. Standard base-protected beta-cyanoethyldiisopropyl phosphoramidites are purchased from

commercial vendors (e.g. PE-Applied Biosystems, Foster City, CA, or Pharmacia, Piscataway, NJ). Non-standard nucleosides are synthesized as per known literature or patented methods. They are utilized as base protected beta-cyanoethyldiisopropyl phosphoramidites.

Oligonucleotides are cleaved from support and deprotected with concentrated NH_4OH at elevated temperature (55-60°C) for 12-16 hours and the released product then dried in vacuo. The dried product is then re-suspended in sterile water to afford a master plate from which all analytical and test plate samples are then diluted utilizing robotic pipettors.

Example 8

Oligonucleotide Analysis - 96 Well Plate Format

The concentration of oligonucleotide in each well is assessed by dilution of samples and UV absorption spectroscopy. The full-length integrity of the individual products is evaluated by capillary electrophoresis (CE) in either the 96 well format (Beckman P/ACEJ MDQ) or, for individually prepared samples, on a commercial CE apparatus (e.g., Beckman P/ACEJ 5000, ABI 270). Base and backbone composition is confirmed by mass analysis of the compounds utilizing electrospray-mass spectroscopy. All assay test plates are diluted from the master plate using single and multi-channel robotic pipettors. Plates are judged to be acceptable if at least 85% of the compounds on the plate are at least 85% full length.

Example 9

Cell culture and oligonucleotide treatment

The effect of antisense compounds on target nucleic acid expression can be tested in any of a variety of cell types provided that the target nucleic acid is present at measurable

levels. This can be routinely determined using, for example, PCR, RNAse protection assay (RPA) or Northern blot analysis. The following four human cell types are provided for illustrative purposes, but other cell types can be routinely used.

T-24 cells:

The transitional cell bladder carcinoma cell line T-24 is obtained from the American Type Culture Collection (ATCC) (Manassas, VA). T-24 cells are routinely cultured in complete McCoy's 5A basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells are routinely passaged by trypsinization and dilution when they reached 90% confluence. Cells are seeded into 96-well plates (Falcon-Primaria #3872) at a density of 7000 cells/well for use in RT-PCR analysis.

For Northern blotting or other analysis, cells may be seeded onto 100 mm or other standard tissue culture plates and treated similarly, using appropriate volumes of medium and oligonucleotide.

A549 cells:

The human lung carcinoma cell line A549 is obtained from the American Type Culture Collection (ATCC) (Manassas, VA). A549 cells are routinely cultured in DMEM basal media (Gibco/Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Gibco/Life Technologies, Gaithersburg, MD), penicillin 100 units per mL, and streptomycin 100 micrograms per mL (Gibco/Life Technologies, Gaithersburg, MD). Cells are routinely passaged by trypsinization and dilution when they reached 90% confluence.

NHDF cells:

Human neonatal dermal fibroblast (NHDF) are obtained from the Clonetics Corporation (Walkersville MD). NHDFs are routinely maintained in Fibroblast Growth Medium (Clonetics Corporation, Walkersville MD) supplemented as recommended by the supplier. Cells are maintained for up to 10 passages as recommended by the supplier.

HEK cells:

Human embryonic keratinocytes (HEK) are obtained from the Clonetics Corporation (Walkersville MD). HEKs are routinely maintained in Keratinocyte Growth Medium (Clonetics Corporation, Walkersville MD) formulated as recommended by the supplier. Cells are routinely maintained for up to 10 passages as recommended by the supplier.

Treatment with antisense compounds:

When cells reached 80% confluency, they are treated with oligonucleotide. For cells grown in 96-well plates, wells are washed once with 200 μL OPTI-MEMJ-1 reduced-serum medium (Gibco BRL) and then treated with 130 μL of OPTI-MEMJ-1 containing 3.75 μg/mL LIPOFECTINJ (Gibco BRL) and the desired oligonucleotide at a final concentration of 150 nM. After 4 hours of treatment, the medium is replaced with fresh medium. Cells are harvested 16 hours after oligonucleotide treatment.

Example 10

Analysis of oligonucleotide inhibition of TGF-ß expression

Antisense modulation of TGF-ß expression can be assayed in a variety of ways known in the art. For example, TGF-ß mRNA levels can be quantitated by Northern blot analysis, RNAse protection assay (RPA), competitive polymerase chain reaction (PCR), or real-time PCR (RT-PCR). RNA analysis can be performed on total cellular RNA or poly(A)+ mRNA. Methods

of RNA isolation are taught in, for example, Ausubel, et al., Current Protocols in Molecular Biology, Volume 1, John Wiley & Sons, Inc., **1993**, pp. 4.1.1-4.2.9 and 4.5.1-4.5.3. Northern blot analysis is routine in the art and is taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 1, John Wiley & Sons, Inc., **1996**, pp. 4.2.1-4.2.9. Real-time quantitative (PCR) can be conveniently accomplished using the commercially available ABI PRISMJ 7700 Sequence Detection System, available from PE-Applied Biosystems, Foster City, CA and used according to manufacturer's instructions. Other methods of PCR are also known in the art.

TGF-ß protein levels can be quantitated in a variety of ways well known in the art, such as immunoprecipitation, Western blot analysis (immunoblotting), ELISA, flow cytometry or fluorescence-activated cell sorting (FACS). Antibodies directed to TGF-ß can be identified and obtained from a variety of sources, such as PharMingen Inc., San Diego CA, or can be prepared via conventional antibody generation methods. Methods for preparation of polyclonal antisera are taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1997**, pp. 11.12.1-11.12.9. Preparation of monoclonal antibodies is taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1997**, pp. 11.4.1-11.11.5.

Immunoprecipitation methods are standard in the art and can be found at, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1998**, pp. 10.16.1-10.16.11. Western blot (immunoblot) analysis is standard in the art and can be found at, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1997**, pp. 10.8.1-

10.8.21. Enzyme-linked immunosorbent assays (ELISA) are standard in the art and can be found at, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 2, John Wiley & Sons, Inc., **1991**, pp. 11.2.1-11.2.22.

Example 11

Poly(A)+ mRNA isolation

Poly(A)+ mRNA is isolated according to Miura et al., *Clin. Chem.*, **1996**, *42*, 1758-1764. Other methods for poly(A)+ mRNA isolation are taught in, for example, Ausubel, et al., *Current Protocols in Molecular Biology*, Volume 1, John Wiley & Sons, Inc., **1993**, pp. 4.5.1-4.5.3. Briefly, for cells grown on 96-well plates, growth medium is removed from the cells and each well is washed with 200 μL cold PBS. 60 μL lysis buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.5 M NaCl, 0.5% NP-40, 20 mM vanadyl-ribonucleoside complex) is added to each well, the plate is gently agitated and then incubated at room temperature for five minutes. 55 μL of lysate is transferred to Oligo d(T) coated 96-well plates (AGCT Inc., Irvine CA). Plates are incubated for 60 minutes at room temperature, washed 3 times with 200 μL of wash buffer (10 mM Tris-HCl pH 7.6, 1 mM EDTA, 0.3 M NaCl). After the final wash, the plate is blotted on paper towels to remove excess wash buffer and then air-dried for 5 minutes. 60 μL of elution buffer (5 mM Tris-HCl pH 7.6), preheated to 70°C is added to each well, the plate is incubated on a 90°C hot plate for 5 minutes, and the eluate is then transferred to a fresh 96-well plate.

Cells grown on 100 mm or other standard plates may be treated similarly, using appropriate volumes of all solutions.

Example 12

Total RNA Isolation

Total mRNA is isolated using an RNEASY 96J kit and buffers purchased from Qiagen Inc. (Valencia CA) following the manufacturer's recommended procedures. Briefly, for cells grown on 96-well plates, growth medium is removed from the cells and each well is washed with 200 μL cold PBS. 100 μL Buffer RLT is added to each well and the plate vigorously agitated for 20 seconds. 100 μL of 70% ethanol is then added to each well and the contents mixed by pipetting three times up and down. The samples are then transferred to the RNEASY 96J well plate attached to a QIAVACJ manifold fitted with a waste collection tray and attached to a vacuum source. Vacuum is applied for 15 seconds. 1 mL of Buffer RW1 is added to each well of the RNEASY 96J plate and the vacuum again applied for 15 seconds. 1 mL of Buffer RPE is then added to each well of the RNEASY 96J plate and the vacuum applied for a period of 15 seconds. The Buffer RPE wash is then repeated and the vacuum is applied for an additional 10 minutes. The plate is then removed from the QIAVACJ manifold and blotted dry on paper towels. The plate is then re-attached to the QIAVACJ manifold fitted with a collection tube rack containing 1.2 mL collection tubes. RNA is then eluted by pipetting 60 μL water into each well, incubating 1 minute, and then applying the vacuum for 30 seconds. The elution step is repeated with an additional 60 μL water.

Example 13

Real-time Quantitative PCR Analysis of TGF-ß mRNA Levels

Quantitation of TGF-ß mRNA levels is determined by real-time quantitative PCR using the ABI PRISMJ 7700 Sequence Detection System (PE-Applied Biosystems, Foster City, CA) according to manufacturer's instructions. This is a closed-

tube, non-gel-based, fluorescence detection system which allows high-throughput quantitation of polymerase chain reaction (PCR) products in real-time. As opposed to standard PCR, in which amplification products are quantitated after the PCR is completed, products in real-time quantitative PCR are quantitated as they accumulate. This is accomplished by including in the PCR reaction an oligonucleotide probe that anneals specifically between the forward and reverse PCR primers, and contains two fluorescent dyes. A reporter dye (e.g., JOE or FAM, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 5' end of the probe and a quencher dye (e.g., TAMRA, obtained from either Operon Technologies Inc., Alameda, CA or PE-Applied Biosystems, Foster City, CA) is attached to the 3' end of the probe. When the probe and dyes are intact, reporter dye emission is quenched by the proximity of the 3' quencher dye. During amplification, annealing of the probe to the target sequence creates a substrate that can be cleaved by the 5'-exonuclease activity of Taq polymerase. During the extension phase of the PCR amplification cycle, cleavage of the probe by Taq polymerase releases the reporter dye from the remainder of the probe (and hence from the quencher moiety) and a sequence-specific fluorescent signal is generated. With each cycle, additional reporter dye molecules are cleaved from their respective probes, and the fluorescence intensity is monitored at regular intervals by laser optics built into the ABI PRISMJ 7700 Sequence Detection System. In each assay, a series of parallel reactions containing serial dilutions of mRNA from untreated control samples generates a standard curve that is used to quantitate the percent inhibition after antisense oligonucleotide treatment of test samples.

PCR reagents are obtained from PE-Applied Biosystems, Foster City, CA. RT-PCR reactions are carried out by adding

25 μL PCR cocktail (1x TAQMANJ buffer A, 5.5 mM $MgCl_2$, 300 μM each of dATP, dCTP and dGTP, 600 μM of dUTP, 100 nM each of forward primer, reverse primer, and probe, 20 Units RNAse inhibitor, 1.25 Units AMPLITAQ GOLDJ, and 12.5 Units MuLV reverse transcriptase) to 96 well plates containing 25 μL poly(A) mRNA solution. The RT reaction is carried out by incubation for 30 minutes at 48°C. Following a 10 minute incubation at 95°C to activate the AMPLITAQ GOLDJ, 40 cycles of a two-step PCR protocol are carried out: 95°C for 15 seconds (denaturation) followed by 60°C for 1.5 minutes (annealing/extension).

Example 14

Antisense inhibition of murine TGF-ß1

Antisense oligonucleotides were designed to hybridize to the mouse TGF-ß1 nucleic acid sequence, using published sequence information (GenBank accession No AJ009862; Locus name MMU009862, provided herein as SEQ ID NO: 1). All oligonucleotides have phosphorothioate backbones and are 2'-methoxyethyl (2'-MOE) gapmers.

Table 1

Antisense oligonucleotides targeted to mouse TGF-ß1

ISIS #	Nucleotide sequence[1] (5'--> 3')	SITE on TARGET SEQUENCE[2]	SEQ ID NO.
105193	**TGTCT**GGAGGATCCG**CGGCG**	49	2
105194	**TGCTC**CTTTGCCGGC**TCCCA**	149	3
105195	**CGAGA**CAGCGCAGTG**CCAAG**	325	4
105196	**GGCTC**CCGAGGGCTG**GTCCG**	435	5
105197	**GCAGG**AGTCGCGGTG**AGGCT**	696	6
105198	**AAAGG**TGGGATGCGG**AGGCC**	801	7
105199	**CAGTA**GCCGCAGCCC**CGAGG**	875	8
105200	**AGTCC**CGCGGCTGGC**CTCCC**	937	9
105201	**GGCTT**CGATGCGCTT**CCGTT**	992	10
105202	**GGCGG**TACCTCCCCC**TGGCT**	1057	11
105203	**CGCCT**GCCACCCGGT**CGCGG**	1119	12
105204	**GTCCA**CCATTAGCAC**GCGGG**	1193	13
105205	**GGCAC**TGCTTCCCGA**ATGTC**	1282	14
105206	**GTCAG**CAGCCGGTTA**CCAAG**	1411	15
105207	**AGTGA**GCGCTGAATC**GAAAG**	1515	16
105208	**GATGG**TGCCCAGGTC**GCCCC**	1598	17
105209	**AGGAG**CAGGAAGGGC**CGGTT**	1627	18
105210	**TCCGG**TGCCGTGAGC**TGTGC**	1680	19
105211	**GCCCT**TGGGCTCGTG**GATCC**	1796	20
105212	**CGCCC**GGGTTGTGTT**GGTTG**	1896	21
105213	**GGCTT**GCGACCCACG**TAGTA**	1969	22
105214	**GGCGG**GGCTTCAGCT**GCACT**	2030	23
110409	**CGCCC**GGGTTGTGCT**GGTTG** 1 base mismatch to 105212	1896	24
110410	**GTGCT**CCCATTGAAA**GCCGG** 8 base mismatch to 105204	1193	25

[1]Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-). All C residues, including 2'-MOE and 2'-deoxy residues, are 5-methyl-cytosines.

[2]Position of first nucleotide at the target site on GenBank accession No AJ009862; Locus name MMU009862, provided herein as SEQ ID NO: 1).

The antisense compounds in the table above were screened by Northern blot at 200 nM oligonucleotide concentration in mouse bEND3 endothelial cells (see Montesano *et al.*, *Cell*, 1990, *62*, 435, and Stepkowski *et al.*, *J. Immunol.*, 1994, *153*, 5336). Cells were treated with oligonucleotide (200 nM) and 10 μg/ml of Lipofectin (Life Technologies, Inc., Gaithersburg, MD) for 4 hours. Cells were then washed and allowed to recover for a further 24 hr. RNA was isolated and TGF-ß1 mRNA expression was measured by Northern blotting. The gels were stripped and reprobed for expression of a housekeeping gene (G3PDH) to confirm equal loading. TGF-ß1 levels are expressed as a percent of control activity, normalized to G3PDH. Results are shown in Table 2.

Table 2

Antisense inhibition of mouse TGF-ß1

ISIS #	% of Control Activity	% Inhibition	SEQ ID NO.
105193	6	94	2
105194	3	97	3
105195	20	80	4
105196	8	92	5
105198	19	81	7
105199	69	31	8
105200	18	82	9
105201	86	14	10

ISIS #	% of Control Activity	% Inhibition	SEQ ID NO.
105202	209	--	11
105203	160	--	12
105204	47	53	13
105205	12	88	14
105206	11	89	15
105207	31	69	16
105208	148	--	17
105209	20	80	18
105211	148	---	20
105212	16	84	21
105213	9	91	22
105214	10	90	23

Oligonucleotides ISIS 105193, 105194, 105195, 105196, 105198, 105200, 105204, 105205, 105206, 105207, 105209, 105212, 105213 and 105214 gave greater than 50% inhibition of TGF-ß1 mRNA in this experiment and are preferred.

Example 15

Dose response of antisense oligonucleotides targeted to murine TGF-ß1

bEND.3 cells were treated with oligonucleotides at various concentrations with 15 μg/ml Lipofectin for 4 hours, then washed and allowed to recover for 24 hours. TGF-ß1 mRNA levels were determined by Northern blot analysis and normalized to G3PDH levels. Results are shown in Table 3.

Table 3

Dose response of antisense oligonucleotides targeted to murine TGF-ß1

Oligonucleotide	**Dose (nM)**	**% of Control**	**% Inhib.**
Lipofectin		100	
ISIS 105195			
	25	47	53
	50	35	65
	100	25	75
	200	18	82
	300	8	92
ISIS 105199			
	25	115	--
	50	126	--
	100	125	--
	200	103	--
ISIS 105204			
	25	31	69
	50	22	78
	100	16	84
	200	11	89
	300	11	89
ISIS 105212			
	25	43	57
	50	29	71
	100	26	74
	200	18	82
	300	24	76
ISIS 105214			
	25	30	70
	50	17	83
	100	17	83
	200	11	89
	300	14	86

ISIS 105195, 105204, 105212 and 105214 had IC50s below 25 nM in this experiment and are preferred.

Example 16

"Humanized" mouse TGF-ß1 antisense oligonucleotide

It was determined by BLAST analysis (Altschul SF et al., *J. Mol. Biol.* **1990**, *215*, 403-10) that ISIS 105204, designed to target mouse TGF-ß1, has only a single mismatch to the human TGF-ß1 gene target, and, except for the 5'-most base on the oligonucleotide, is complementary to a site beginning at nucleotide 1167 on the human target (GenBank accession no. X02812; locus name HSTGFB1; Derynck,R., et al., **1985**, *Nature* 316, 701-705). An oligonucleotide (**TTCCA**CCATTAGCAC**GCGGG**; ISIS 113849; SEQ ID NO: 26) was designed and synthesized which was a complete match to the human target sequence at this site. This compound is a phosphorothioate backbone with 2'-MOE nucleotides shown in bold. All C residues are 5-methyl C.

Example 17

Efficacy of ISIS 105204 in rat kidney cells

ISIS 105204, designed to target mouse TGF-ß1, was tested in rat NRK kidney cells (available from American Type Culture Collection, Manassas VA). This oligonucleotide has 100% complementarity to the rat TGF-ß1 sequence (GenBank accession no.X52498; locus name RNTGFB1, provided herein as SEQ ID NO: 27). A dose response is shown in Table 4. ISIS 105195, which is targeted to a region of the mouse TGF-ß1 sequence which shares only 9 of 20 nucleobases with the rat sequence, is shown for comparison.

Table 4

Dose response of antisense oligonucleotides targeted to mouse TGF-ß1 in rat NRK cells

ISIS #	Dose (nM)	% of Control activity	% Inhibition	SEQ ID NO
Lipofectin		100	--	
105195				4
	100	115	--	
	200	98	2	
	300	97	3	
105204				13
	50	56	44	
	100	50	50	
	200	39	61	

Example 18

Effect of antisense inhibition of TGF-ß1 on fibrotic scarring

A model for fibrosis has been developed in which osmotic pumps are implanted subcutaneously in rats. Normally the pump becomes encapsulated by fibrotic scar tissue. The effect of antisense inhibition of TGF-ß1 on scarring can be analyzed and quantitated.

2 ml Alzet osmotic pumps (Alza corporation, Palo Alto, CA) were implanted subcutaneously on the back of female Sprague Dawley rats. Four rats per experimental group were implanted with pumps containing PBS, 5 mg of TGF-ß1 antisense oligonucleotide and 5 mg of an eight base mismatch control oligonucleotide, ISIS 110410. After 3 weeks the encapsulation tissue surrounding the pump was removed, weighed, snap frozen, and evaluated for TGF-ß1 mRNA by Northern blot analysis or RNAse protection assay using the rCK3b template (Pharmingen, San Diego CA) and by immunohistochemistry. For the latter, formalin fixed, paraffin embedded tissues were stained with Masson's Trichrome Stain for Collagen and immunochemical

localization of oligonucleotide. Frozen tissues were antibody-stained for TGF-ß1 (antibody from Santa Cruz Biotechnologies, Santa Cruz, CA), and EDA Fibronectin (antibody from Harlan Bioproducts, Sussex, England). The antibodies were detected with secondary reagents directly conjugated to HRP and DAB (brown) was used as the substrate.

TGF-ß1 expression in the scar tissue was reduced by greater than 50% after 28-day treatment with ISIS 105204 oligonucleotide dose 15 mg/kg; and to greater than 30% with a dose of 5 mg/kg), as measured by Northern blot analysis of TGF-ß1 mRNA levels. This is shown in Table 5.

Table 5

Effect of ISIS 105204 on TGF-ß1 expression in rat scar tissue

Oligonucleotide	% of control	% inhibition	SEQ ID NO
Saline control	100	--	
ISIS 110410 (8-base mismatch of 105204)	83	17	25
ISIS 105204	44	56	13

Immunohistochemical staining showed that TGF-ß1 protein expression is reduced in scar tissue from mice treated with ISIS 105204. Levels of collagen and fibronectin, which are markers for fibrosis, were also reduced in scar tissue from these mice. Staining also showed a decrease in the number of CD18 positive cells.

Example 19

Antisense oligonucleotides targeted to human TGF-ß1

Antisense oligonucleotides were designed to hybridize to the human TGF-ß1 nucleic acid sequence, using published sequence information; Derynck,R., et al., 1985, Nature 316, 701-705; GenBank accession number X02812; locus name HSTGFB1,

incorporated herein as SEQ ID NO: 28. Oligonucleotides have phosphorothioate backbones and are 2'MOE gapmers. Sequences are shown in Table 6.

Table 6

Antisense oligonucleotides targeted to human TGF-ß

ISIS #	**Nucleotide sequence[1] (5'--> 3')**	**SITE on TARGET SEQUENCE[2]**	**SEQ ID NO:**
104978	**CGACT**CCTTC**CTCCGCTCCG**	113	29
104979	**CTCGT**CCCTC**CTCCCGCTCC**	209	30
104980	**AAGTC**CTGCC**TCCTCGCGGG**	317	31
104981	**AAGGG**TCTAG**GATGCGCGGG**	531	32
104982	**CTCAG**GGAGA**AGGGCGCAGT**	692	33
104983	**GCACT**GCCGA**GAGCGCGAAC**	802	34
104984	**GTAGC**AGCAG**CGGCAGCAGC**	862	35
104985	**ATGGC**CTCGA**TGCGCTTCCG**	968	36
104986	**GCGTA**GTAGT**CGGCCTCAGG**	1136	37
104987	**ACCAC**TGCCG**CACAACTCCG**	1447	38
104988	**TCGGC**GGCCG**GTAGTGAACC**	1557	39
104989	**GAAGT**TGGCA**TGGTAGCCCT**	1788	40
104990	**GGCGC**CCGGG**TTATGCTGGT**	1875	41
104991	**CTCCA**CCTTG**GGCTTGCGGC**	1956	42
104992	**AATGA**CACAG**AGATCCGCAG**	2155	43
104993	**TAGAT**CTAAC**TACAGTAGTG**	2305	44
104994	**CGCCT**GGCCT**GAACTACTAT**	2525	45
104995	**CCCAG**GCTGG**TCTCAAATGC**	2609	46

[1]Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-). All C residues, including 2'-MOE and 2'-deoxy residues, are 5-methyl-cytosines.

[2]Position of first nucleotide at the target site on GenBank accession number X02812; locus name HSTGFB1, provide herein as SEQ ID NO: 28.

Oligonucleotides were screened in 293T human kidney cells at a concentration of 200 nM with 10 μg/ml of Lipofectin for a period of four hours. Cells were washed and allowed to recover for a further 24 hr. At this point RNA was isolated and TGF-ß1 mRNA levels were determined by Ribonuclease Protection Assay (RPA) using the hCK-3 template (Pharmingen, San Diego CA) according to the manufacturer's instructions. TGF-ß1 mRNA levels were normalized to GAPDH and expressed as a percentage of untreated control. Results are shown in Table 7.

Table 7

Antisense Inhibition of Human TGF-ß1

ISIS #	% of Control Activity	% Inhibition	SEQ ID NO:
104978	150	--	29
104979	94	6	30
104980	82	18	31
104981	86	14	32
104982	94	6	33
104983	52	48	34
104985	59	41	36
104986	59	41	37
104987	63	37	38
104988	71	29	39
104989	86	14	40
104990	57	43	41
104991	52	48	42
104992	47	53	43
104993	84	16	44
104994	60	40	45
104995	64	36	46
105204	23	77	13

In this experiment ISIS 104983, 104985, 104986, 104990, 104991, 104992, 104994 and 105204 gave at least 40% inhibition of human TGF-ß1 mRNA and are preferred. ISIS 104992 and 105204 gave over 50% inhibition.

Example 20

Dose responses of antisense oligonucleotides targeted to human TGF-ß1

ISIS 113849, 105204, 110410 (8 base mismatch of 105204) and 104992 were tested at 50, 100 and 200 nM for ability to inhibit TGF-ß1 mRNA levels. Results are shown in Table 8.

Table 8

Dose response of oligonucleotides targeted to human TGF-ß1

ISIS #	Dose (nM)	% of Control Activity	% Inhibition	SEQ ID NO:
104992	50	65	35	43
	100	68	32	
	200	93	7	
105204	50	31	69	13
	100	16	84	
	200	23	77	
110410	50	85	15	25
	100	64	36	
	200	50	50	
113849	50	29	71	26
	100	24	76	
	200	36	64	

ISIS 105204 and 113849 had IC50s below 50 nM in this experiment. These oligonucleotides were found to have little effect on TGF-ß2 or TGF-ß3 mRNA levels.

Example 21

Antisense compounds targeted to murine TGF-ß2

Antisense oligonucleotides were designed to hybridize to the mouse TGF-ß2 nucleic acid sequence, using published sequence information from GenBank accession number X57413; Miller,D.A., et al., *Mol. Endocrinol.* **1989**, 3, 1108-1114; locus name MMTGFB2, incorporated herein as SEQ ID NO: 47. The oligonucleotides are shown in Table 9.

Table 9

Antisense sequences targeted to murine TGF-ß2

ISIS #	Nucleotide sequence[1] (5'--> 3')	SITE on TARGET SEQUENCE[2]	SEQ ID NO.
104996	**GCCGG**CAGTTTCAGC**AGCTC**	34	48
104997	**CTCGC**ACCCTTCCCT**AGCTT**	259	49
104998	**TTTCT**TGCTCCAGGC**GGCCA**	362	50
104999	**GAGCA**GGCGGCGAGG**ATCCC**	493	51
105000	**GCCCT**GCCTTCCACA**CGTGT**	671	52
105001	**GTGCG**GAGTGGCTGA**TCTGA**	830	53
105002	**AAAAT**GCAACGCGTT**CCCAA**	1016	54
105003	**CCGGG**ACCAGATGCA**GGAGC**	1247	55
105004	**TCCGG**CTTGCCTTCT**CCTGC**	1451	56
105005	**GGGTT**TTGCAAGCGG**AAGAC**	1668	57
105006	**CGATG**TAGCGCTGGG**TGGGA**	1754	58
105007	**GGTCT**TCCCACTGGT**TTTTT**	2032	59
105008	**AAGCT**TCGGGATTTA**TGGTG**	2321	60
105009	**ACCGT**GATTTTCGTG**TCCTG**	2478	61
105010	**GCGGG**CTGGAAACAA**TACGT**	2854	62
105011	**CCCCT**GGCTTATTTG**AGTTC**	3075	63
105012	**ACCGG**CTTGCTTAAA**CTGGC**	3297	64
105013	**CAGCC**ACTTCACGGT**CAAAA**	3352	65
105014	**ATGGA**CCCAGGTAGC**TCATG**	3753	66

ISIS #	Nucleotide sequence[1] (5'--> 3')	SITE on TARGET SEQUENCE[2]	SEQ ID NO.
105015	**CACCC**GCCACATGAC**TCACA**	3874	67
105016	**TACAC**CCCATGAGCA**CCAAA**	4097	68

[1]Emboldened residues, 2'-methoxyethoxy- residues (others are 2'-deoxy-). All C residues, including 2'-MOE and 2'-deoxy residues, are 5-methyl-cytosines.

[2]Position of first nucleotide at the target site on GenBank accession number X57413; Miller,D.A., et al., *Mol. Endocrinol.* **1989**, 3, 1108-1114; locus name MMTGFB2, incorporated herein as SEQ ID NO: 47.

The oligonucleotides shown in Table 9 were screened for the ability to inhibit mouse TGF-ß2 mRNA expression by ribonuclease protection assay (RPA) in mouse R6 +/+ fibroblast cells. Cells were treated with oligonucleotide (200 nM) and 10 μg/ml of Lipofectin (Life Technologies, Inc.) for 4 hours. Cells were then washed and allowed to recover for a further 24 hours. RNA was isolated and TGF-ß2 mRNA expression was measured by RPA using the mCK3b template (Pharmingen, Inc., San Diego CA) according to manufacturer's directions. Results were normalized to GAPDH and expressed as a percent of RNA levels in untreated control cells. Results are shown in Table 10.

Table 10

Antisense inhibition of mouse TGF-ß2 mRNA expression

ISIS #	% of control activity	% inhibition	SEQ ID NO:
104996	83	17	48
104997	79	21	49
104998	70	30	50

ISIS #	**% of control activity**	**% inhibition**	**SEQ ID NO:**
104999	90	10	51
105000	64	36	52
105001	37	63	53
105002	51	49	54
105003	28	72	55
105004	52	48	56
105005	77	23	57
105006	53	47	58
105007	60	40	59
105008	55	45	60
105009	28	72	61
105010	27	73	62
105011	48	52	63
105012	35	65	64
105013	40	60	65
105014	43	57	66
105015	64	36	67
105016	89	11	68

ISIS 105001, 105002, 105003, 105004, 105006, 105008, 105009, 105010, 105011, 105012, 105013 and 105014 gave at least about 45% inhibition of TGF-ß2 mRNA expression in this experiment and are preferred. Of these, ISIS 105003, 105009 and 105010 gave at least 70% inhibition.

Interestingly, it was found that oligonucleotides that reduced TGF-ß2 also reduced TGF-ß3 mRNA levels. This is shown in Table 11.

Table 11

Common inhibition of murine TGF-ß2 and TGF-ß3 by antisense oligonucleotides targeted to murine TGF-ß2

ISIS #	% inhibition of TGF-ß2	% inhibition of TGF-ß3	SEQ ID NO:
104996	17	3	48
104997	21	11	49
104998	30	--	50
104999	10	14	51
105000	36	16	52
105001	63	48	53
105002	49	23	54
105003	72	67	55
105004	48	49	56
105005	23	20	57
105006	47	60	58
105007	40	29	59
105008	45	23	60
105009	72	55	61
105010	73	49	62
105011	52	57	63
105012	65	42	64
105013	60	55	65
105014	57	43	66
105015	36	52	67
105016	11	12	68

Example 22

Reduction in peritoneal adhesions by antisense inhibition of TGF-ß1

The surface of the peritoneal cavity and the enclosed organs are coated with a layer of mesothelial cells that are easily damaged by injury or infection. Following injury (surgery, for example), adhesions form which cause permanent scarring. This scarring can result in bowel obstruction, pain, and/or female infertility. A rat model for peritoneal adhesions has been developed (Williams et al., **1992**, *J. Surg. Res. 52*, 65-70). Animal models have demonstrated that TGF-β promotes the formation of postoperative pelvic adhesions.

In these experiments bilateral uterine injuries were created in 250 gm Sprague Dawley rats by cautery, scraping and crushing. Rats then received 5 mg (20 mg/kg) of an antisense oligonucleotide (ISIS 105204), 5 mg of a scrambled control oligonucleotide (ISIS 110410) or 1 mL of saline vehicle via intraperitoneal injection.

Uterine adhesions were then graded by masked evaluators using a clinical scale of 0-3 on days 3, 7 and 14 after injury.

In order to localize the target tissue of the antisense oligonucleotides, an additional group of rats were injected with a reporter oligonucleotide and biopsies were perfomed on the uterus, liver and kidney of the treated animals. The tissues were then fixed and the reporter oligonucleotide was immunolocalized with a specific antibody. The reporter oligonucleotide was concentrated heavily in the area of uterine injury at 2 hours and persisted in uterine cells at 72 hours indicating that the oligonucleotide does localize to the injured area.

A single dose of the antisense oligonucleotide (ISIS 105204) to TGF-β1 significantly reduced the severity of peritoneal adhesions, from a mean of 3.0 for control animals

to 1.2 for antisense treated animals. A scrambled control oligonucleotide (ISIS 110410) gave a mean adhesion score of 2.4 over the entire study.

Example 23

Effect of antisense inhibition of TGF-ß1 on lung fibrosis

A model of lung fibrosis has been developed using bleomycin to induce pulmonary fibrosis in mice. Wild, JS,. SN Giri et al., 1996, Exp. Lung Res. 22, 375-391. Mice receive an intratracheal dose of bleomycin (0.125U/mouse) or saline, followed by treatment with antisense oligonucleotide (i.p.) over 2 weeks. Mice were treated with ISIS 105204 or 110410. RNA was isolated from lungs and TGF-ß1 mRNA levels were determined for mice treated with saline or bleomycin alone, saline or bleomycin plus ISIS 105204, and bleomycin plus the scrambled control ISIS 110410. Results are shown in Table 12. These studies showed a significant reduction of bleomycin-induced lung hydroxyproline content, prolyl hydroxylase and lipid peroxidation. Lung histopathology showed fibrotic lesions to be reduced in bleomycin treated animals receiving the TGF-B1 oligonucleotide compared to saline or mismatch treated animals. Also, RPA (ribonuclease protection assay) analysis revealed a 45% reduction in TGF-β1 RNA in animals treated with ISIS 105204.

Table 12

Effect of antisense inhibition of TGF-ß1 on lung fibrosis

Treatment	% of control	% inhibition
Saline	100	--
Saline + 105204	81	19
Bleomycin	70	30
Bleomycin + 110410	73	27
Bleomycin + 105204	37	63

Example 24

Effect of antisense inhibition of TGF-ß1 on conjunctival scarring

Animal models for a variety of fibrotic diseases and conditions exist. Conjunctival scarring is a major predictor of visual prognosis in a variety of eye conditions, including post-surgical healing. For example, the most common cause of failure of glaucoma filtration surgery is scarring at the bleb and sclerostomy sites. A model of conjunctival scarring in the mouse eye has been developed to investigate potential determinants, modes of prevention and treatments for conjunctival scarring. Reichel et al., **1998**, *Br. J. Ophthalmol. 82,* 1072-1077. This model is used to evaluate the effects of locally or systemically delivered antisense to TGF-ß on conjunctival scarring. Alternatively, antisense compounds can be administered at the time of trabeculectomy filtration surgery.

Study #1

Animals were anesthetized, and the general glaucoma filtration trabeculectomy procedure was followed. A conjunctival flap was raised and a viscoelastic solution (e.g., Healon) was injected into the anterior chamber. A paracentesis stab incision was made using a 75 Beaver blade

into the anterior chamber. A sclerotomy was performed through the paracentesis incision using a membrane punch and a peripheral iridectomy was done through the sclerostomy. The conjunctival flap was repositioned and closed with suture in two layers. Oligonucleotide solution (100 μl of 40 μM in the case of rabbits, less in mouse or rat) was injected into the bleb by tunneling a 30 gauge needle through the conjunctiva adjacent to the bleb. Animals were sacrificed 24 hours after treatment and eyes were fixed and examined histologically for collagen, fibronectin, and immunohistochemically for TGF-ß.

In this study, Balb-c mice, a highly inbred strain of mice used to produce monoclonal antibodies, were randomly allocated to one of five treatment groups; subconjunctival injection (5µl) of 25 µg or 12.5 µg of either a TGF-β1 antisense oligonucleotide (ISIS 105204) or a scrambled control oligonucleotide (ISIS 110410), or the carrier saline control. Cellular distribution of oligonucleotide in glaucoma surgery was assessed following subconjunctival administration of a reporter oligonucleotide into the filtration bleb immediately after surgery in NZW rabbits. Mice and rabbits were assessed clinically and enucleated eyes were analyzed at set time intervals histologically.

At days 3 and 7 mouse eyes (n=4) showed significantly reduced white cell infiltration and collagen fibril deposition in the TGF-β1 oligonucleotide treated groups compared to controls. There was also a significant decrease in localization of fibroblasts and elastin related fibers on days 3,7 and 14 in groups treated with the TGF-β1 antisense oligonucleotide.

At 7 days mouse eyes (4 eyes/treatment group) showed significantly reduced ($p<0.05$) conjunctival scar formation in the TGF-β1 treated animals as compared to the control group.

Study #2

Forty-eight New Zealand White rabbits (Charles River UK Ltd; 2-2.4 kg, 12-14 weeks old) were used following an acclimatization period of around 5 days. All rabbits underwent glaucoma drainage surgery with one of 6 treatments, which were randomly assigned. Of these, 18 rabbits were sacrificed at 14 days post surgery and the rest were observed until 30 days. The study was performed as a randomized, controlled study with masked observers. Animals were randomly assigned to 6 treatment groups as shown below.

Table 13

Group	Oligonucleotide (OGN) and target	SEQ ID NO	Dose	Volume	No. animals 30 days	No. animals 14 days
1	ISIS 105204 (TGF-β1)	13	100 µg	100 µl	5	3
2	ISIS 123285 (TGF-β2)	69	100 µg	100 µl	5	3
3	ISIS 123787 (TGF-βRII)	70	100 µg	100 µl	5	3
4	ISIS 124189 (CTGF)	71	100 µg	100 µl	5	3
5	Missense (ISIS 10410)	25	100 µg	100 µl	5	3
6	PBS	-	-	100 µl	5	3

ISIS 123285 has the sequence 5'-CCGTGACCAGATGCAGGATC-3'. ISIS 123787 has the sequence 5'-GGCCAGGGAGCTGCCCAGCT-3'. ISIS 124189 has the sequence 5'-GCCAGAAAGCTCAAACTTGA-3'. These are all targeted to murine sequences. In all three sequences, all internucleoside linkages are phosphorothioates, all cytosines are replaced with 5-methylcytosine, and positions 1-5 and 16-20 are substituted with 2'-MOE.

The oligonucleotides were administered immediately pre- and postoperatively (i.e. on Day 0) to the operated eye of each rabbit by subconjunctival injection. A 25G needle was placed on the same site in each eye 5 mm behind the limbus at the nasal margin of the superior rectus muscle, such that a visible bleb was formed in the supranasal quadrant of each eye. The contralateral eye was used as a control.

The method described by Cordeiro et al. (*Invest. Opthalmol. Vis. Sci.* **38**:1639-1646, 1997; Cordeiro et al., *Invest. Opthalmol. Vis. Sci.* **40**:2225-2234, 1999) was used. All rabbits underwent filtration surgery to the left eye only. A partial thickness 8-0 silk corneal traction suture (Ethicon, Edinburgh, Scotland) was placed superiorly and the eye pulled down. A fornix based conjunctival flap was raised following which a blunt dissection of subconjunctival space was performed to a distance of 15 mm behind the limbus.

An MVR blade was used to make a partial thickness scleral incision 4 mm behind the limbus and a scleral tunnel to the corneal stroma was fashioned. A 22 G/25mm Venflon 2 intravenous cannula was passed through a scleral tunnel anteriorally until the cannula needle was visible in the clear cornea. Entry into the anterior chamber was made with the cannula needle which was then withdrawn as the cannula was advanced to the mid-pupillary area. The cannula was trimmed and bevelled at its scleral end so that it protruded 1 mm from the insertion point and a 10-0 nulon suture was used to fix the tube to the scleral surface. The conjunctival incision was closed with two interrupted sutures and a central mattress-type 10-0 nulon suture on a B/V 100-4 needle (Ethicon) to give a water-tight closure. One drop of atropine sulfate 1% and betnesol N ointment was instilled at the end of surgery.

All animals were checked ophthalmologically at baseline and every day for the first 3 days after surgery and

thereafter every third or fourth day until Day 30, as described below. Ophthalmological assessment included intraocular pressure, bleb size, bleb vascularity, anterior chamber depth and anterior chamber activity.

Measurement of intraocular pressure in both eyes was made using the Mentor tonopen. This was performed after topical installation of 0.4% benoxinate HCl local anesthetic. Three recordings per eye were made per time point and a mean reading was documented.

Bleb width, height and length was measured and bleb area (width and length) was calculated. Measurements were made with a microsurgical caliper in mm.

Bleb and conjunctival vascularity was performed by dividing the conjunctival area into quadrants: superior, nasal and temporal. Each quadrant was then assessed and recorded using color photography. Grading was performed as follows: 0=avascular, +1=normal vascularity, +2=hyperemic, +3=very hyperemic. Avascularity was also assessed and graded binomially as follows: 1=presence of avascularity in any area of the eye, 0=no vascularity.

Anterior chamber depth was assessed subjectively, graded and recorded as either deep (=+2), shallow (=+1) or flat (=0).

Anterior chamber activity (inflammation) was assessed by slit lamp photography and graded as follows: 0=no cells, +1=cells present, +2=fibrin formation, +3=a hypopyon.

A general macroscopic description was recorded on the injected area in terms of complications such as lid edema, chemosis, hemorrhage and corneal toxicity.

The primary efficacy endpoint was taken as bleb survival. Bleb failure was defined as the appearance of a flat, vascularized, scarred bleb in association with a deep anterior chamber. Kaplan-Meier and log rank statistics were used to compare treatment groups. The multivariate analysis of variance (ANOVA) was used to compare differences between

treatments and effects of time and treatment, using the SPSS package and the Bonferroni correction. Bleb area and height were analyzed using the repeated measures procedure by the Generalized Linear Model (SPSS). This allowed comparison of treatment groups over the whole study period using the tests of between-subjects. Anterior chamber depth and activity were assessed using the General Linear Model, as described above. Analysis of conjunctival vascularity changes was performed using GLM statistics as described above for superior, temporal and nasal quadrants. Avascularity was assessed using Pearson's Chi-Squared test to compare treatment groups.

At the beginning of the study, all rabbits receiving treatment with Group 3 antisense oligonucleotide developed endopthalmitis within 3 days of surgery. A very heavy growth (confluent) of *Staphylococcus aureus* was isolated from these cases which were sacrificed in accordance with the protocol and Home Office regulations. As the cause of the infection was isolated, and the causative batch of oligonucleotides identified, a new treatment group was substituted and treated with a fresh batch of stringently tested Group 3 antisense oligonucleotide. There was one case of death following administration of the anesthetic but prior to surgery (rabbit 42).

A Kaplan-Meier bleb survival curve was constructed and is shown in Figure 1, with the mean survival in each treatment group shown in Table 14. Survival was prolonged in TGF-β2 and TGF-βIIR antisense oligonucleotide groups (mean survival of 19.4 and 16.5 days, respectively) compared to the TGF-β1, CTGF and control groups. Treatment with the TGF-β2 antisense oligonucleotide significantly prolonged bleb survival compared to TGF-β1 (log rank $p=0.0009$), CTGF ($p=0.0042$), missense ($p=0.0072$) oligonucleotide and PBS control ($p=0.0035$) treatment groups. Compared to PBS control, TGF-β2 antisense

oligonucleotide increased bleb survival by 5.68 days, and TGF-βIIR antisense oligonucleotide increased bleb survival by 2.78 days.

Table 14

Group	TGF-β2 OGN	TGF-βIIR OGN	TGF-β1 OGN	CTGF OGN	Missense OGN	PBS control
Mean survival (days)	19.4	16.5	14.88	14.2	14.37	13.72
Median survival (days)	17.0	17.0	16.0	14.0	14.0	14.0

Analysis of intraocular pressure showed no significant difference between treatment groups and at any time point. Bleb area and height were analyzed using the repeated measures procedure by the Generalized Linear Model (GLM). Bleb area and height were analyzed using the repeated measures procedure by the Generalized Linear Model (SPSS). Comparison of treatment groups over the whole study period revealed no significant difference between treatment groups with respect to bleb area or bleb height. Anterior chamber depth was assessed using GLM as described above. TGF-β2 oligonucleotide treated eyes had shallower anterior chamber depth compared to the other treatments. Comparison between treatment groups showed a significant difference (p=0.034). Figure 2 shows the mean grade of anterior chamber depth in the operated eye over the study period. GLM was also used to compare anterior chamber inflammation between treatment groups, and was found to be significantly different (p=0.02). Vascularity was graded in each quadrant and analyzed using GLM statistics as described above. Comparison of treatment groups for each

quadrant showed no significant difference throughout the study period. However, at day 1, the PBS control group was significantly more vascular than the other treatment groups in the superior and temporal quadrants.

The TGF-β2 antisense oligonucleotide significantly prolonged bleb survival by 5.68 days after perioperative application in glaucoma filtration surgery in the rabbit. Bleb survival was pre-defined as the primary efficacy endpoint of the study. Although bleb survival was also prolonged with the TGF-βIIR antisense oligonucleotide group, this did not reach statistical significance when compared to control. Treatment with the TGF-β2 antisense oligonucleotide significantly prolonged bleb survival compared to TGF-β1 (log rank p=0.0009), CTGF (p=0.0042), missense (p=0.0072) and PBS control (p=0.0035) treatment groups.

No difference was noted between treatment groups with regard to intraocular pressure. This may be due to a breakdown in the blood-aqueous barrier producing destabilization of intraocular pressure. Evidence of increased aqueous outflow through the filtration site in 6B1 treatment groups compared to controls may also be obtained from measurements of anterior chamber depth and bleb morphology. Thus, shallow anterior chambers and higher and larger blebs indicate improved outflow. Shallow anterior chambers were observed for longer periods of time in the TGF-β2 antisense oligonucleotide treatment group.

No difference between treatment groups was recorded in relation to conjunctival vascularity and anterior chamber activity over the whole study period. This parameter is important in assessing the safety of the injected substances. If local tolerance was present, it would be seen clinically as increased vascular injection and uveitis (increased anterior chamber activity). It is interesting to note, however, that

at day 1, the PBS control group was significantly more vascular than the other treatment groups, suggesting that all test substances were in fact very well-tolerated and perhaps even anti-inflammatory compared to the PBS control.

The results show that TGF-β2 antisense oligonucleotide treatment is effective in reducing the conjunctival scarring response following glaucoma filtration surgery in a model of aggressive scarring. Despite the relatively small numbers of animals in the treatment group, the fact that statistical significance in bleb survival was achieved, shows the potency of antisense oligonucleotides to TGF-β2. In addition, the oligonucleotide appears to be well tolerated *in vivo*, with no evidence of adverse reactions. These results show that TGF-β2 is an effective and sage anti-scarring therapeutic agent.

The cellular profile suggested that TGF-β1 oligonucleotide delayed the development of the wound healing response. Immunohistochemical staining with an antibody specific for the reporter oligonucleotide in rabbit eyes revealed intense and localized staining of the TGF-β1 oligonucleotide to fibroblasts, epithelial cells and macrophages in the sclera and conjunctiva at the surgical site.

Example 25

Effect of antisense inhibition of TGF-ß1 on inflammation-human skin xenograft model in the SCID mouse.

Another model used to investigate the processes of inflammation and scarring involves the use of SCID mice transplanted with human skin. SCID mice lack an enzyme necessary to fashion an immune system and can therefore be converted into a model of the human immune system when injected with human cells or tissues. In these experiments human skin (2 cm^2) from various surgical procedures (breast

reductions or neonatal foreskin) or from cadavers was transplanted onto the side of SCID mice with sutures or surgical staples. After four to six weeks, the mice were bled and tested for Ig to ensure the SCID lineage. After 8 to 10 weeks, the xenograft skin was treated with antisense oligonucleotide,. ISIS 105204, SEQ ID NO: 13) in a cream formulation at 48, 24, and 4 hours prior to the injection of 4000U of tumor necrosis factor-alpha (TNF-α). Levels of TGF-β protein were then assayed in the epidermis and dermis of the xenograft skin by immunohistochemical staining 24 hours after TNF-α injection. Levels were reported as a percentage of the area showing positive staining for the presence of TGF-β protein.

In the epidermis, 3% of the area showed positive staining after treatment with TGF-β antisense oligonucleotide relative to basal levels of 50% and levels of 37% for the placebo cream group. This data was shown to be statistically significant (P=0.0001).

In the dermis, TGF-β levels were below 0.5% with basal levels at 2.5% and placebo cream group levels of 2%.